ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

Founded 1924

Acta Pathologica et Microbiologica Scandinavica is intended for the prompt publication of original research in the fields of pathology, microbiology, and immunology. It is included in Current Contents, Excerpta Medica, and Medlars.

Acta Pathologica et Microbiologica Scandinavica is a nonprofit-making scientific journal. Since 1924, it has been published by the Scandinavian Societies for Medical Microbiology and Pathology. It appears in three sections: Section A: Pathology, Section B: Microbiology, and Section C: Immunology.

Acta Pathologica et Microbiologica Scandinavica has subscribers in more than seventy countries throughout the world with a wide readership in the major research institutes, hospitals, laboratories, and specialist libraries.
Usually, only articles submitted by Scandinavian authors will be accepted, but the Editorial Board may invite contributions from authors outside Scandinavia.
Submission of a manuscript for publication in this Journal will be held to imply that the work is original, that it has not been published elsewhere, and that, if accepted, it will not be published in any other journal, without the Editor's written permission.
Contributions should usually be in English, but papers in French or German can also be accepted (with English summaries).
The Editorial Board takes no responsibility for contents of or views implied or expressed by the authors or advertisements.

EDITORIAL CORRESPONDENCE

All communications regarding manuscripts and editorial matters should be addressed to the Editorial Office, c/o Inger Danielsen, Secretary, Johnstrups Allé 6, DK-1923 Copenhagen V, Denmark.

SUBSCRIPTION

At present one annual volume of Section A, one of Section B, and one of Section C (each section consisting of 6 issues) will contain a total of approximately 1600 pages. During the past few years approximately seven free supplements have been issued annually. The subscription price is:

Sect. A, B and C:	D.kr. 700.– plus postage D.kr. 40.–	($ 132.10, £ 57.35, DM 324.50)
Sect. A:	D.kr. 420.– plus postage D.kr. 15.–	($ 77.65, £ 33.70, DM 190.75)
Sect. B:	D.kr. 420.– plus postage D.kr. 15.–	($ 77.65, £ 33.70, DM 190.75)
Sect. C:	D.kr. 280.– plus postage D.kr. 10.–	($ 51.80, £ 22.50, DM 127.20)

Back numbers (*whole volumes or single copies*) are available.
NB. All prices are subject to exchange rate fluctuation.

All business communications regarding subscriptions, distribution, changes of address, *advertisements, or orders* of back numbers should be addressed to MUNKSGAARD, International Publishers Ltd., 35 Nörre Sögade, DK-1370 Copenhagen K, Denmark.

The symbol on cover is designed by the Norwegian architect Jacob Grundt.

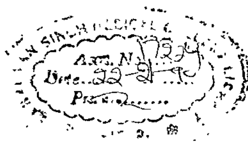
ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

Section C IMMUNOLOGY

VOL. 83 C. FASC. 1–6 1975

MUNKSGAARD COPENHAGEN

Published by the
Scandinavian Societies for Microbiology and Pathology



Distributed by Munksgaard
International Booksellers and Publishers, Ltd.
35 Norre Sögade, DK-1370 Copenhagen K, Denmark

PRINTED IN DENMARK
VALD. PEDERSENS BOGTRYKKERI A-S
COPENHAGEN

INDEX

VOL. 83 C. FASC. 1–6. 1975

Acta path. microbiol. scand. Sect. C, *83:* 1–14, 1975

ALLO-AGGRESSION IN CHICKENS

Serological Identification of the Major Genetic Locus

MORTEN SIMONSEN

The University Institute for Experimental Immunology

Simonsen, M. Allo-aggression in chickens Serological identification of the major genetic locus. Acta path. microbiol. scand. Sect. C, *83:* 1–14, 1975.

The B-group is the conventional name for the major histocompatibility system (MHS) of the chicken and was first described as a blood group locus. It actually comprises an unknown number of linked loci, at least one of which (AA) governs allo-aggression as manifested, and studied here by the GVH reaction. The gene product(s) of the AA locus would appear to be expressed not only in T cells but also for example in the nucleated erythrocytes, which fact would then in principle permit their detection by haemagglutination. In practice such detection requires that a number of other haemagglutinating antibodies, directed against other antigens of the same B haplotype be first removed, because the unabsorbed antiserum against one given haplotype seems to agglutinate the RBC of almost any unrelated chicken in the world. A strategy is described which allows a planned absorption of the irrelevant specificities from sera against entire haplotypes; hence lead to successful typing for the AA locus. Some observations suggest an extreme degree of linkage disequilibrium in White Leghorns with respect to the genes determining the B1 haplotype, which appears to be a common haplotype in that particular race.

Key words: Allo-aggression; major histocompatibility system; chicken.

M. Simonsen, The University Institute for Experimental Immunology, Nørre Allé 71, DK-2100 Copenhagen Ø, Denmark.

Received 7.xii.74 Accepted 7.xii.74

Introduction to Terminology

Contrary to the possibly first thought of the reader, this paper is not an investigation of cock fights. The work concerns aggression at the cellular level, the aggressive cells being T lymphocytes from one chicken, and the target as well as the stimulus for aggression being cells of another chicken (though not necessarily T cells). The term of allo-aggression was used first by *Niels Jerne* (1971) to cover the cellular reactions which can result from the encounter of allogeneic lymphocytes both *in vivo* and *in vitro*, as exemplified by studies of graft versus host (GVH) and mixed lymphocyte culture (MLC) reactions. Two important features of these reactions, which seem to be pure T cell reactions, are a) the high frequency of responding cells to a foreign haplotype of the major histocompatibility system (MHS) of the species (*Simonsen* 1967, *Wilson et al.* 1968, *Bach et al.* 1969, *Atkins* & *Ford* 1974), and b) the low factor of immunization shown by the fact that virgin cells react about as fast and as well as specifically primed cells (*Simonsen* 1962, *Ford* & *Simonsen* 1971). An additional feature of these reactions mentioned by *Jerne* is several observations to the

effect that they are either not, or to a very much smaller degree provoked by xenogeneic cells. This latter claim has later been challenged in the case of MLC reactions (*Widmer et al.* 1972, *Nielsen* 1972). On the other hand, the widespread occurrence even among invertebrates of reactions which seem to be very analogous to transplant rejections (*Hildemann* 1974), as well as the largely negative attempts to inhibit GVH or MLC reactions with antibodies to known Ig determinants (*Crone et al.* 1972), suggest two more reasons for having a separate term for these reactions. The term of allo-aggression seems to me apt, also because it avoids the word immune with its acquired connotation of an adaptive mode of responsiveness mediated by immunoglobulins.

I consequently find it reasonable to suggest that the term of AA (allo-aggression) locus be used for the component(s) of the MHS-genome which is responsible for allo-aggression. The genetic nomenclature to be used in this paper will refer to the whole of the MHS-genome as the *B*-system (rather than the traditional *B*-locus, since it manifestly is a complex of several loci in analogy with H-2 and HL-A). Hence the *AA* locus is part of the *B*-system, and AA antigens (AA1, AA2, etc.) are gene products which form part of the corresponding haplotypes (B1, B2, etc.). Genotypic designations are in italics and are given numerial superscripts. Thus a $B^{1/2}$ chicken will have cells which may express the B1 and B2 haplotypes more or less fully in different cell types.

Introduction to the Principal Findings

Increasingly, the MHS becomes a pole of attraction towards which converge such problems in immunology which have not found a satisfactory solution elsewhere.

Thus, the MHS is credited with a central role in the genetic control of immunological responsiveness in both delayed hypersensibility and in antibody production (*McDevitt & Landy* 1972), and with presumably related roles in types of cellular cooperation which are basic to the immune response, such as operation between T and B cells (*Katz et* 1973), and between macrophages and T (*Shevach & Rosenthal* 1973). In mice MHS has been found to determine resist to oncogenic viruses (*Lilly et al.* 1964), in man different HL-A haplotypes are f to correlate with the occurrence of an al impressive variety of diseases, rapidly c increase (*Svejgaard et al.* 1975). Not forgotten are of course the more fa roles to be expected from genes in a M namely that they specify gene produc extreme importance in most kinds of t plantation.

It is crucial to further understandin the complicated interrelationship betw the functions of the MHS that we sh obtain a much better definition of its g products. Complete structural definitio still a distant aim, but a better serolog definition may be within more rapid re And this is exactly where the chicken app to have something to offer, although genetic mapping of its MHS has hardly begun.

The main substance of the present pa is a large-scale investigation verifying, further analysing an earlier report (*Simon* 1973) that it is possible to develop typing s which will permit selection of such ind duals of an outbred population which terms of GVH reactivity are compatible w a given recipient. In other words, allo-aggr sion is avoidable by appropriate serology.

MATERIALS AND METHODS

Animals

The birds of this study comprise both inbr and outbred chickens. The former were 2 sublin of the fairly highly, though not completely inbr lines of White Leghorn-like chickens which we started originally in Iowa and later inbred furth by Dr. *L. W. Schierman* and colleagues in Ne York. Newly hatched batches of the $B^{1/1}$ an $B^{2/2}$ lines were kindly supplied by Drs. *L. W Schierman* and *R. A. McBride* in January, 196 and have been kept here by random matings withi the lines. Also ($B^{1/1} \times B^{2/2}$) F1, and F2 hybrid have been employed. The parents of the $B^{1/1}$ batcl

we originally received were known to segregate for the blood group loci *D* and *L*, for each of which there are two known alleles, designated *1* and *2*. The parents of the $B^{2/2}$ chicks were, in contrast, all homozygous $D^{2/2}$ and $L^{2/2}$. The two lines are very unlikely to be segregating for any other blood group system, at least not for anyone detectable by the haemagglutination techniques used in Dr. *Schierman's* or in my own hands.

The outbred birds have mostly been White Leghorns (W.L.) from a commercial Danish hatchery from which they were obtained as fertilized eggs. Some W.L.s came from a different Danish firm. Finally some outbred New Hampshires and Faverolles from a private pen were employed.

Immunization

Inbred $B^{1/1}$ *and* $B^{2/2}$ birds were injected with blood cells from $B^{1/2}$ F1 hybrids for the production of anti-B2 and anti-B1 sera respectively. Washed blood cells from 2.5 ml of citrated full-blood were injected i.v. once a week for 6-8 weeks, and the sera were harvested 5-7 days after the last injection. They were kept stored at –20° C.

Haemagglutination

Haemagglutinating antibodies were detected by an open glass-slide agglutination technique using 2 per cent suspensions of washed RBC in phosphate buffered saline (PBS) and serum dilutions in PBS, one drop of each. Unless there was already gross agglutination after 5–10 min, a drop of 20 per cent of normal chicken serum in PBS was added and the final reading made 30 min later. Sometimes this addition would cause instantaneous massive agglutination where none was seen beforehand. (This curious phenomenon was first observed with a purified IgG fraction which had practically no agglutinating power of its own. It has often been marked also with antisera which had first undergone absorption procedures, or had been frozen or thawed repeatedly. This agglutination-enhancing principle in normal serum is not itself an Ig, or a general albumen effect. Its more exact nature is being studied separately).

Typing sera for the D and L specificities were obtained from Dr. *Schierman*.

GVH Splenomegaly

The degree of GVH spleen enlargement in chicken embryos injected with immunocompetent T cells of an appropriate genotype is a well-known measure of allo-aggression. In this study embryos were injected i.v. on day 13–14 of incubation, and were killed 6 days later for removal and weighing of the spleens. The inoculum consisted of WBC from citrated blood, separated from the RBC by mild centrifugation, and then spun down and washed thrice in TCM 199 supplemented with 2 per cent BSA. Final suspensions in the diluent were adjusted to 3×10^7 WBC/ml. Each specimen of test cell suspension was tested in a group of usually 5 eggs. The results are given in the Tables as the mean of the log mg spleen weights recorded.

The GVH Inhibition Test and Its Use in Tissue Typing

This is the key method of the present investigation. It is based on the fact that the GVH splenomegaly can be quantitatively suppressed by alloantibodies directed against some antigen or other which form part of the MHS (or B-group) of the donor cell (*McBride et al.* 1966, *Crone et al.* 1972). Equal volumes of cell suspension and of antiserum dilution (usually 1:100 in PBS) have been mixed and pre-incubated for 1 hour at 37° C before injection.

The present use of GVH-inhibition as a typing procedure requires the further step of absorbing the inhibitory serum prior to its use as described above. Any kind of cell which can specifically remove the inhibitory allo-antibody by absorption must itself contain a relevant antigen, either identical, or very similar to that of the test cells. The actual absorptions have been performed with the cell sediment of citrated whole blood washed three times. This is of course a vast excess of RBC relative to WBC, but the exact amount of WBC present is immaterial, since we have found that the absorbing capacity of red and white blood cells are about equal cell per cell. Usually 1 volume of serum dilution was absorbed twice with half a volume of packed, washed blood cells, leaving the mixture at room temperature for 20–30 min for each absorption.

RESULTS

1. *The Panagglutinating Properties of the Antisera*

Immunization of a given bird with blood from a random donor usually yields an antiserum which gives a strong haemagglutination, not only with the RBC of the donor, but also with RBC of most other birds. This is a fact of long standing (*Todd* 1930). Later work identified several unlinked systems including the important "B-locus" (*Briles et al.* 1950) which was also identified later on as the major genetic site of control of GVH reactions (*Jaffe* & *McDermid* 1962, *Schierman* & *Nordskog* 1963).

1*

effect that they are either not, or to a very much smaller degree provoked by xenogeneic cells. This latter claim has later been challenged in the case of MLC reactions (*Widmer et al.* 1972, *Nielsen* 1972). On the other hand, the widespread occurrence even among invertebrates of reactions which seem to be very analogous to transplant rejections (*Hildemann* 1974), as well as the largely negative attempts to inhibit GVH or MLC reactions with antibodies to known Ig determinants (*Crone et al.* 1972), suggest two more reasons for having a separate term for these reactions. The term of allo-aggression seems to me apt, also because it avoids the word immune with its acquired connotation of an adaptive mode of responsiveness mediated by immunoglobulins.

I consequently find it reasonable to suggest that the term of AA (allo-aggression) locus be used for the component(s) of the MHS-genome which is responsible for allo-aggression. The genetic nomenclature to be used in this paper will refer to the whole of the MHS-genome as the *B*-system (rather than the traditional *B*-locus, since it manifestly is a complex of several loci in analogy with H-2 and HL-A). Hence the *AA* locus is part of the *B*-system, and AA antigens (AA1, AA2, etc.) are gene products which form part of the corresponding haplotypes (B1, B2, etc.). Genotypic designations are in italics and are given numerial superscripts. Thus a $B^{1/2}$ chicken will have cells which may express the B1 and B2 haplotypes more or less fully in different cell types.

Introduction to the Principal Findings

Increasingly, the MHS becomes a pole of attraction towards which converge such problems in immunology which have not found a satisfactory solution elsewhere.

Thus, the MHS is credited with a central role in the genetic control of immunological responsiveness in both delayed hypersensibility and in antibody production (*McDevitt* & *Landy* 1972), and with presumably related roles in types of cellular cooperation which are basic to the immune response, such as cooperation between T and B cells (*Katz et al.* 1973), and between macrophages and T cells (*Shevach* & *Rosenthal* 1973). In mice the MHS has been found to determine resistance to oncogenic viruses (*Lilly et al.* 1964), and in man different HL-A haplotypes are found to correlate with the occurrence of an already impressive variety of diseases, rapidly on the increase (*Svejgaard et al.* 1975). Not to be forgotten are of course the more familiar roles to be expected from genes in a MHS, namely that they specify gene products of extreme importance in most kinds of transplantation.

It is crucial to further understanding of the complicated interrelationship between the functions of the MHS that we should obtain a much better definition of its gene products. Complete structural definition is still a distant aim, but a better serological definition may be within more rapid reach. And this is exactly where the chicken appears to have something to offer, although the genetic mapping of its MHS has hardly yet begun.

The main substance of the present paper is a large-scale investigation verifying, and further analysing an earlier report (*Simonsen* 1973) that it is possible to develop typing sera which will permit selection of such individuals of an outbred population which in terms of GVH reactivity are compatible with a given recipient. In other words, allo-aggression is avoidable by appropriate serology.

MATERIALS AND METHODS

Animals

The birds of this study comprise both inbred and outbred chickens. The former were 2 sublines of the fairly highly, though not completely inbred lines of White Leghorn-like chickens which were started originally in Iowa and later inbred further by Dr. *L. W. Schierman* and colleagues in New York. Newly hatched batches of the $B^{1/1}$ and $B^{2/2}$ lines were kindly supplied by Drs. *L. W. Schierman* and *R. A. McBride* in January, 1968 and have been kept here by random matings within the lines. Also ($B^{1/1} \times B^{2,2}$) F1, and F2 hybrids have been employed. The parents of the $B^{1/1}$ batch

we originally received were known to segregate for the blood group loci *D* and *L*, for each of which there are two known alleles, designated *1* and *2*. The parents of the $B^{2/2}$ chicks were, in contrast, all homozygous $D^{2/2}$ and $L^{2/2}$. The two lines are very unlikely to be segregating for any other blood group system, at least not for anyone detectable by the haemagglutination techniques used in Dr. *Schierman*'s or in my own hands.

The outbred birds have mostly been White Leghorns (W.L.) from a commercial Danish hatchery from which they were obtained as fertilized eggs. Some W.L.s came from a different Danish firm. Finally some outbred New Hampshires and Faverolles from a private pen were employed.

Immunization

Inbred $B^{1/1}$ *and* $B^{2/2}$ birds were injected with blood cells from $B^{1/2}$ F1 hybrids for the production of anti-B2 and anti-B1 sera respectively. Washed blood cells from 2.5 ml of citrated full-blood were injected i.v. once a week for 6-8 weeks, and the sera were harvested 5-7 days after the last injection. They were kept stored at –20° C.

Haemagglutination

Haemagglutinating antibodies were detected by an open glass-slide agglutination technique using 2 per cent suspensions of washed RBC in phosphate buffered saline (PBS) and serum dilutions in PBS, one drop of each. Unless there was already gross agglutination after 5–10 min, a drop of 20 per cent of normal chicken serum in PBS was added and the final reading made 30 min later. Sometimes this addition would cause instantaneous massive agglutination where none was seen beforehand. (This curious phenomenon was first observed with a purified IgG fraction which had practically no agglutinating power of its own It has often been marked also with antisera which had first undergone absorption procedures, or had been frozen or thawed repeatedly. This agglutination-enhancing principle in normal serum is not itself an Ig, or a general albumen effect. Its more exact nature is being studied separately).

Typing sera for the D and L specificities were obtained from Dr. *Schierman*.

GVH Splenomegaly

The degree of GVH spleen enlargement in chicken embryos injected with immunocompetent T cells of an appropriate genotype is a well-known measure of allo-aggression. In this study embryos were injected i.v. on day 13–14 of incubation, and were killed 6 days later for removal and weighing of the spleens. The inoculum consisted of WBC from citrated blood, separated from the RBC by mild centrifugation, and then spun down and washed thrice in TCM 199 supplemented with 2 per cent BSA. Final suspensions in the diluent were adjusted to 3×10^7 WBC/ml. Each specimen of test cell suspension was tested in a group of usually 5 eggs. The results are given in the Tables as the mean of the log mg spleen weights recorded.

The GVH Inhibition Test and Its Use in Tissue Typing

This is the key method of the present investigation. It is based on the fact that the GVH splenomegaly can be quantitatively suppressed by allo-antibodies directed against some antigen or other which form part of the MHS (or B-group) of the donor cell (*McBride et al.* 1966, *Crone et al.* 1972). Equal volumes of cell suspension and of antiserum dilution (usually 1:100 in PBS) have been mixed and pre-incubated for 1 hour at 37° C before injection.

The present use of GVH-inhibition as a typing procedure requires the further step of absorbing the inhibitory serum prior to its use as described above. Any kind of cell which can specifically remove the inhibitory allo-antibody by absorption must itself contain a relevant antigen, either identical, or very similar to that of the test cells. The actual absorptions have been performed with the cell sediment of citrated whole blood washed three times. This is of course a vast excess of RBC relative to WBC, but the exact amount of WBC present is immaterial, since we have found that the absorbing capacity of red and white blood cells are about equal cell per cell. Usually 1 volume of serum dilution was absorbed twice with half a volume of packed, washed blood cells, leaving the mixture at room temperature for 20–30 min for each absorption.

RESULTS

1. *The Panagglutinating Properties of the Antisera*

Immunization of a given bird with blood from a random donor usually yields an antiserum which gives a strong haemagglutination, not only with the RBC of the donor, but also with RBC of most other birds. This is a fact of long standing (*Todd* 1930). Later work identified several unlinked systems including the important "B-locus" (*Briles et al.* 1950) which was also identified later on as the major genetic site of control of GVH reactions (*Jaffe & McDermid* 1962, *Schierman & Nordskog* 1963).

TABLE 1. *Haemagglutination Reactions with B1-anti-B2 Serum and Three Panels of Unrelated Donors*

W.L.		(W.L. × $B^{1/1}$)F1			N.H. & Fr.	
(a) no.	Serum dilution 1:4	(b) no.	Serum dilution 1:2	Serum dilution 1:10	(c) no.	Serum dilution 1:10
3161	+ +	2896	+ +	+	N.H.6267	+ +
3173	+	3001	+ +	+ +	6268	+ +
3175	+ +	3006	+ +	+ +	6269	+ +
3183	+ +	3004	+ +	+ +	6270	+ +
3187	+ +	3011	+ +	+ +	6271	+ +
3191	+	3028	+ +	+ +	6272	+ +
3194	—	3030	+ +	+ +	6273	+ +
3209	+ +	3039	+ +	+ +	6274	+ +
3541	+ +	3040	+ +	+ +	6275	+ +
3543	+ +	3073	+ +	+ +	6276	+ +
3546	+	3074	+ +	+ +	6277	+ +
3549	+ +	3077	+ +	+ +	6278	+ +
3555	+	3079	+ +	+ +	6279	+ +
3565	+ +	3081	+ +	+ +	6280	+ +
3575	+ +	3143	+ +	(+)	Fr 6281	+ +
		3191	+ +	+ +	6282	+
		3199	+ +	+ +	6283	+ +
		3255	+ +	+ +	6284	+ +
		3263	+ +	(+)	6285	+
		3267	+ +	+ +		
		3269	+ +	+		
		3277	+ +	+ +		
$B^{1/1}$	—	$B^{1/1}$	—	—	$B^{1/1}$	—
$B^{2/2}$	+ +	$B^{2/2}$	+ +	+ +	$B^{2/2}$	+ +

W.L. = White Leghorn. N.H. = New Hampshire. Fr. = Faverolles.
The W.L. mothers of the F1 hybrids of group (b) were ten birds which had been selected by serotyping as not carrying the AA2 allele. Yet each of the maternal haplotypes inherited by the offspring apparently caused strong reactions with the unabsorbed anti B2 serum.

The question here is to what extent the B specificities of anti-B-group sera raised, B1-anti-B2 and B2-anti-B1, cross-react with RBC of unrelated birds after appropriate absorption with F2 cells to removal of D and C specificities. The answer is seen for the anti-B2 serum from the data in Table 1, which contains 3 groups of unrelated donors: (a) 15 White Leghorns which differed by both haplotypes of the MHS from the B2 haplotype since none of the birds contained the AA2 allele (see later), (b) 20 birds which were the F1 offspring of 10 W.L. hens and one $B^{1/1}$ cock. Nor did these hens contain the AA2 allele, (c) 14 New Hampshires and 5 Faverolle birds, also being AA2 negative. Although none of these unrelated birds possessed the whole of the B2 haplotype, since they all lacked the AA2 allele, apparently all but one (W.L. 3194) nevertheless contained at least one of the determinants by which the B2 haplotype differs from the B1 haplotype.

Also the other B-group serum, B2-anti-B1, showed a nearly complete cross-reactivity with panels of unrelated birds. In conclusion, therefore, our two standard anti B-group sera were very nearly panagglutinating, which fact made meaningful typing look rather hopeless at first sight. We shall nevertheless proceed to show how this same fact can be turned into an advantage.

TABLE 3. *AA-phenotyping of White Leghorns by Serology and by GVH Inhibition*

Bird no.	AA1		AA2	
	Serotyping	GVH-typing*	Serotyping	GVH-typing*
3348	—	1.32 ± 0.04	+ +	2.13 ± 0.10
3402	—	1.40 ± 0.05	—	1.28 ± 0.02
3404	—	1.34 ± 0.05	+ +	2.31 ± 0.09
3430	—	1.34 ± 0.07	+ +	2.17 ± 0.21
3444	+ +	1.76 ± 0.09	—	1.39 ± 0.08
$B^{1/1}$	+ +	1.89 ± 0.06	—	1.33 ± 0.03
$B^{2/2}$	—	1.27 ± 0.08	+ +	2.06 ± 0.09

* The presence of AA1 antigen (and of AA2, mutatis mutandis) is revealed in a given bird by the ability of its blood cells to remove the inhibitory antibody from a standard B2-antigen-B1 serum so that the $B^{1/1}$ cells remain uninhibited in their production of splenomegaly. Control absorptions are performed with $B^{1/1}$ and $B^{2/2}$ cells. Results are expressed as the mean log mg spleen weight ± s.e.

TABLE 4. *Summary of Phenotyping for the AA1 and AA2 Antigens in Unrelated Birds*

Exp. no.		Breed	No. of birds	No. of positives by Sero-typing	No. of positives by GVH-typing	"False" positives
AA1	118	W.L.(I)	15	2	2	0
AA1	121	W.L.(II)	5	1	1	0
AA1	128	W.L.(II)	8	6	5	1
AA1	147	W.L.(II)	3	0	0	0
AA1	166	W.L.(II)	14	4	4	0
AA1	168	W.L.(II)	20	4	4	0
AA1	174	W.L.(II)	18	5	4	1
AA1	179	W.L.(II)	21	5	5	0
AA1	201	W.L.(II)	5	1	1	0
AA1	207	W.L.(II)	15	1	1	0
AA1	205	Fr.	5	0	0	0
AA1	205	N.H.	14	11	0	11
AA2	118	W.L.(I)	15	3	3	0
AA2	121	W.L.(II)	5	1	0	1
AA2	132	W.L (II)	8	0	0	0
AA2	147	W.L.(II)	3	0	0	0
AA2	176	W.L.(II)	21	3	0	3
AA2	201	W.L.(II)	5	3	3	0
AA2	206	W.L.(II)	15	0	0	0
AA2	204	Fr.	5	1	0	1
AA2	204	N.H.	14	2	0	2

W.L.(I) and W.L.(II) = White Leghorns from 2 different commercial stocks. N.H. = New Hampshire. Fr. = Faverolles.

that the unabsorbed sera from which they originated were nearly panagglutinating, it was hardly to be expected that absorption with a small panel, essentially chosen at random, apart from the fact that it was known from the GVH test not to contain the AA allele to be defined, would remove all "irrelevant" specificities. Presumably, sooner or later an unrelated bird would agglutinate, and not in fact be a carrier of the presump-

TABLE 5. *AA-typing of* ($B^{1/1} \times B^{2/2}$)*F2 Hybrids by Serology and by GVH Inhibition*

F2 donor for absorption	AA* genotype	GVH inhibition test for AA1	
		AA1	AA2
		log mg ± s.e.	log mg ± s.e.
no.			
377	*1/1*	1.95 ± 0.12	1.31 ± 0.04
476	*1/1*	2.01 ± 0.10	1.26 ±0.06
485	*1/1*	1.98 ± 0.05	1.26 ± 0.04
482	*1/2*	2.02 ± 0.10	2.11 ± 0.13
478	*1/2*	1.92 ± 0.05	2.03 ± 0,05
487	*1/2*	1.94 ± 0 01	2.07 ± 1.13
468	*1/2*	1.95 ± 0.07	2.01 ± 0.10
489	*1/2*	1.98 ± 0.06	2.01 ± 0.11
491	*1/2*	1.97 ± 0.06	1.98 ± 0.17
381	*1/2*	1.87 ± 0.11	2.00 ± 0.03
379	*1/2*	2.07 ± 0.07	1.97 ± 0.04
375	*2/2*	1.17 ± 0 03	2.09 ± 0.09
—ab§		2.11 ± 0.05	2.06 ± 0.11
+ab§		1.26 ± 0.02	1.27 ± 0.04

See legend to Table 3.
§ab = unabsorbed B1-anti-B2, or B2-anti-B1 serum, functions as positive control for GVH inhibition in typing for AA2 and AA1, respectively.
*AA genotype established prior to the GVH inhibition test by AA1 and AA2 typing sera.

tive allele of the AA locus. This indeed happened occasionally, and these are the "false" positives in Table 4, last column. Whenever a "false" positive was investigated further (and most of them were) its blood cells were found to remove the misleading antibody specificity from the serum without removing the antibodies which agglutinated those birds which were proved by the GVH inhibition test to contain the AA allele in question, i.e. the "true" positive. Hence, "false" specificities apparently represented antibodies to antigens of little or no importance for allo-aggression, which had happened to be absent in the absorption panel. It is quite striking that while no more than 2 "false" positives was found in the AA1-typing among a total of 104 White Leghorns comprising 27 "true" positives, there were no fewer than 11 out of 14 New Hampshires to be classified as "false" positives. There is little doubt that this result is connected with the fact that the absorption panel itself consisted always of White Leghorns (actually from both sources I & II), but did not include New Hampshires.

Much more important than the occurrence of some "false" positives is of course the fact that there was not a single bird which in the GVH-inhibition test proved to contain the AA1 or AA2 allele and yet not had its RBC agglutinated by the respective typing serum; in other words, there were no "false" negatives. This fact is basic to the hypothesis, that the allo-aggression locus in birds is not merely linked to blood group loci in the MHS, but that its gene products are themselves haemagglutinogens.

4. *Experiments with F2 Hybrids*

In this section evidence will be provided in support of two necessary predictions from the postulate that allo-aggression is determined by a locus which forms part of the MHS (the "B-system"). These are: (a) that ($B^{1/1} \times B^{2/2}$) F2 hybrids should behave in the GVH inhibition test in accordance with

TABLE 6. *Identical Segregation in* ($B^{1/1} \times B^{2/2}$)*F2 Hybrids of AA Antigens and of Other Antigens of the MHS*

AA* genotype of F2 birds	No. of birds per genotype	Agglutination pattern of a B1-anti-B2 serum absorbed with 6 individual White Leghorns phenotyping AA2 and tested against 2 per cent RBC suspensions of 27 individual F2 birds					
		W.L. numbers					
		84	95	99	129	134	135
2/2	4	+ +	+ +	+ +	+ +	+ +	+ +
1/2	20	+ +	+ +	+ +	+ +	+ +	+ +
1/1	3	—	—	—	—	—	—

* Genotype established as in Table 5.

TABLE 7. *Agglutination Data after Absorption of a B2-anti-B1 Serum with Blood from White Leghorns*

B2-anti-B1 serum absorbed with RBC of individual W.L.s (AA type)	RBC Suspensions from same panel of White Leghorns										
	AA1-positive					AA1-negative					
	1343	1480	1256	1487	3011	1329	1336	1337	1341	1477	$B^{1/1}$
W.L.1343 (1)	—	—	—	—	—	—	—	—	—	—	—
W.L.1480 (1)	—	—	—	—	—	—	—	—	—	—	—
W.L.1256 (1)	—	—	—	—	—	—	—	—	—	—	—
W.L.1487 (1)	—	—	—	—	—	—	—	—	—	—	—
W.L.3011 (1)	—	—	—	—	—	—	—	—	—	—	—
W.L.1329 (not 1)	+ +	+ +	+ +	+ +	+ +	—	—	—	—	—	+ +
W.L.1336 (not 1)	+ +	+ +	+ +	+ +	+ +	+ +	—	—	—	—	+ +
W.L.1337 (not 1)	+ +	+ +	+ +	+ +	+ +	+ +	+	—	—	—	+ +
W.L.1341 (not 1)	+ +	+ +	+ +	+ +	+ +	+ +	+	+	—	+	+ +
W.L.1477 (not 1)	+ +	+ +	+ +	+ +	+ +	+ +	+	+	+	—	+ +

-typing as being either AA1, AA2, or ..th, and (b) that the "irrelevant" serological specificities of the B-system segregate together with the AA specificities.

The first of these predictions is supported by the experiment in Table 5 which shows complete accordance of sero-typing and GVH-typing in twelve F2 hybrids.

The second prediction is met by the haemagglutination results in Table 6 where twenty-seven F2 hybrids were typed both for *AA and for some "irrelevant" specificities of* the B2-haplotype The latter were detected by the antibodies which remained in a B1-anti-B2 serum absorbed with 6 individual W.L.s which had each been typed as AA2. Since these removed all antibodies which could inhibit the GVH reaction, it seems *justified to* consider the remaining haemagglutinins irrelevant to a lo-aggression.

The linkage between the various genes of the MHS which determine haemagglutinogens (be they relevant or not to allo-aggression) was studied also by a different *approach. This* was to absorb anti-B1 and anti-B2 sera with B-system heterozygotes of the F2 generation and *to check the absorbed* sera for presence of possibly unabsorbed specificities by means of F1 hybrid erythrocytes. None were found after individual absorption with eight F2 heterozygotes.

5. *The B-system Haplotypes in Inbred and Outbred Birds Compared by Serological Means*

The results in the previous section showed no signs of crossing over, albeit in small numbers of segregating B-system hybrids. We shall now turn to the much less predictable matter

TABLE 8. *Search for Serological Differences between the B1 Haplotypes of* $B^{1/1}$ *Inbreds and of AA1-positive Outbreds*

AA1-positive producers of anti-$B^{1/2}$ sera	Agglutination of RBC suspensions of origin: $B^{1/1}$ ($D^{2/2}$, $L^{2/2}$)		$B^{2/2}$ ($D^{2/2}$, $L^{2/2}$)	
	Serum unabsorbed	Serum absorbed*	Serum unabsorbed	Serum absorbed*
W.L. 52	++	—	++	—
W.L. 235	++	—	++	—
W.L. 241	++	—	++	—
W.L. 275	++	—	++	—
W.L. 1266	++	—	++	—
W.L. 1268	++	—	++	—
W.L. 1274	+	—	++	—

The sera were raised by a course of 6 i.v. injections over 2 months. The washed blood cells from 2 ml of $B^{1/2}$ blood was given per injection. The sera were harvested 6 days later, and were both absorbed and tested in dilutions 1:2 in PBS. *Absorptions were performed with $B^{2/2}$ ($D^{2/2}$, $L^{2/2}$) blood cells as described under Methods.

of genetic association in outbred populations. With the available material there were two obvious and connected questions which could be approached experimentally:

(a). To what extent are the antibody specificities of, say, a B2-anti-B1 serum raised in inbreds removable by absorption with blood of AA1-positive outbred W.L.s? (Or likewise for B1-anti-B2?).

(b). Will outbreds of type AA1, or AA2 give rise to antibodies, when immunized with blood from $B^{1/1}$ and $B^{2/2}$ inbreds respectively?

The joint answer to both questions suggests that the B2 haplotype is different in inbreds and outbreds, while the B1 haplotype proved to be not demonstrably different.

The findings re (a). All out of eighteen outbred W.L.s (from both sources) typing as AA1 could remove all agglutinins to $B^{1/1}$ erythrocytes from various B2-anti-B1 sera. A sample of such data (Table 7) show also that absorption with five birds which were not AA1 left agglutinins which predictably agglutinated $B^{1/1}$ cells as well as all AA1-positive outbred cells, but otherwise had a variable agglutination pattern. In contrast, as shown already in Table 6, none out of six outbred W.L.s typing AA2 could remove all agglutinins to B2 from a B1-anti-B2 serum. A subsidiary finding from checkerboard absorption and haemagglutination experiments with these six birds was the fact that the "irrelevant" agglutinins were of at least two different specificities.

The findings re (b). Table 8 shows the haemagglutination data with sera drawn from seven W.L.s typing AA1 but not AA2 and immunized with blood cells from a ($B^{1/1} \times B^{2/2}$) F1 donor. All unabsorbed sera agglutinated as expected $B^{2/2}$ cells, but they also agglutinated $B^{1/1}$ cells sharing the same D2 and L2 antigens. Unfortunately, there were no control cells available of $B^{1/1}$ ($D^{1/1}$, $L^{1/1}$) genotype. However, the absorption with $B^{2/2}$ ($D^{2/2}$, $L^{2/2}$) cells removed all agglutinin to the $B^{1/1}$ cells, too. The part played in Table 8 by the D2 and L2 antigens is still uncertain. The only safe conclusion is that whatever the seven W.L.s may have seen as foreign in the inbred B1 haplotype, it must have been specificities shared with the B2 haplotype, hence governed by a segment of the B-system chromosome which is not revealed in cross-immunization between inbred $B^{1/1}$ and $B^{2/2}$.

Table 9 shows in contrast to Table 8 that AA2-positive W.L.s immunized with $B^{1/2}$ blood from an F1 hybrid produced antibodies to $B^{2/2}$ cells which were not all removed by absorption with $B^{1/1}$ cells. The fact that they were truely directed against determinants of

TABLE 9. *Search for Serological Difference Between the B2 Haplotypes of Inbreds and of AA2-positive Outbreds*

AA2 positive producers of anti-$B^{1/2}$ sera	Agglutination of RBC suspensions of origin: $B^{1/1}$		$B^{2/2}$	
	Serum unabsorbed	Serum absorbed*	Serum unabsorbed	Serum absorbed*
W.L. 95	+ +	—	+ +	+ +
W.L. 99	+ +	—	+ +	+ +
W.L. 134	+ +	—	+ +	+ +
W.L. 135	+ +	—	+ +	+ +

* Sera were absorbed with RBC from $B^{1/1}$, as well as from B1-homozygous F2 hybrids to removal of L and D specificities in addition to B1-specificities. This should leave only antibodies specific for determinants of the B2 haplotype. The sera were raised by a course of injections similar to exp. of Table 8.

the inbred B2 haplotype was born out in additional tests with a panel of twelve F2 hybrids. Thus the B2 haplotype of the inbred birds *cannot* be identical with the haplotype(s) present in the outbred W.L.s, although they share the same AA gene product, as far as the evidence from GVH inhibition has indicated.

6. *The AA Antigens in Inbred and Outbred Birds Studied by Attempts to Inhibit Outbred Cells by Antisera Raised in Inbreds*

The experiment in this section (Table 10) asks basically the same question as we started with, namely the question of to what extent the anti-B1 and anti-B2 sera raised in inbreds will *cross-react with cells from outbreds*. However, instead of looking at haemagglutination (as in Table 1), we will turn the attention to T cells and ask to what extent the sera can inhibit the T cells of outbred birds in their own GVH reaction? Moreover, since we now know a way to phenotype the outbreds with respect to AA1 and AA2, we can refine the question and ask whether the anti-B1 and anti-B2 sera, in cases where they do inhibit outbred T cells, will then conform to the simplest expectation and kindly inhibit such cells only which come from donors containing the respective AA antigen?

Once again the fact [illegible] not symmetrical: While anti-B2 seems [illegible]nhibit only cells from AA2-positive birds, anti-B1 *does* in 4 out of 4 cases also inhibit cells which are AA1-negative, and highly significantly so. It is important to realise that any inhibition caused by these sera is almost with certainty due to one or other gene of the B-system. Firstly, strongly haemagglutinating antisera to D and L locus antigens are quite inert in GVH inhibition (unpublished, but solid data). Secondly, the inhibition of AA1-negative donors by a $B^{2/2}$-anti-$B^{1/1}$ serum was not removed by absorption with a pool of RBC from eight F2 hybrids of the $B^{2/2}$ genotype, as was to be expected, unless the relevant specificities were in fact directed to antigens of the B1 haplotype.

7. *The AA Antigens in Inbred and Outbred Birds Studied by the Direct in vivo Compatibility Test*

In this final section we shall briefly judge the AA typing of outbreds by the "Consumers Test" to which one would no doubt submit the typing, if birds were priceless creatures to be rescued from a state of immunodeficiency by means of transplantation. In other words, we want to know whether the typing will in fact permit the transplantation of immunocompetent T cells without causing severe GVH reaction as a sign of allo-aggression.

The preliminary communication (*Simonsen* 1973, Table 2) already presented some

TABLE 10. *Inhibition with B1-anti-B2, and B2-anti-B1 Sera of GVH Reactions Produced with Outbred Donor Cells*

WBC donor no.	AA phenotype*	PBS	Incubation of WBC with anti-B1, 1:100	anti-B2, 1:100
W.L. 26	not 1, or 2	2.11 ± 0.02	1.42 ± 0.10	2.33 ± [illegible]
W.L. 28	not 1, or 2	1.66 ± 0.14	1.13 ± 0.05	1.74 ± [illegible]
W.L. 30	not 1, or 2	2.24 ± 0.11	1.21 ± 0.08	1.97 ± [illegible]
W.L. 32	not 1, or 2	2.23 ± 0.06	1.28 ± 0.05	2.00 ± [illegible]
W.L. 34	1, not 2	1.87 ± 0.06	1.19 ± 0.03	2.07 ± [illegible]
$B^{1/1}$	1	1.82 ± 0.06	1.06 ± 0.07	not done
W.L. 84	2, and 1	2.43 ± 0.08	not done	1.37 ± [illegible]
W.L. 99	2, not 1	2.60 ± 0.03	not done	1.31 ± [illegible]
W.L. 135	2, not 1	2.31 ± 0.07	not done	1.10 ± [illegible]
$B^{2/2}$	2	1.98 ± 0.12	not done	1.19 ± [illegible]

GVH inhibition performed as described in Methods, except that the donor cells are from outbred [illegible] Control cells are $B^{1/1}$ and $B^{2/2}$. Figures are mean log spleen weight ± s.e. *AA-phenotypes [illegible] prior to this experiment, both by sero-typing and by GVH-typing.

TABLE 11. *GVH Reactions with WBC from Outbred W.L.s Injected into Inbred* [illegible] *and* [illegible] *Embryos*

WBC donor no.	AA phenotype*	Embryos $B^{1/1}$ $\bar{X}1$ ± s.e.	$B^{2/2}$ $\bar{X}$ ± s.e.	$\bar{X}2$ - [illegible]
W.L. 42	not 1, or 2	2.25 ± 0.09	2.29 ± 0.05	[illegible]
W.L. 46	not 1, or 2	2.06 ± 0.07	2.17 ± 0.06	[illegible]
W.L. 62	not 1, or 2	2.25 ± 0.04	2.25 ± 0.07	[illegible]
W.L. 254	not 1, or 2	2.19 ± 0.05	2.25 ± 0.05	[illegible]
W.L. 273	not 1, or 2	2.06 ± 0.12	2.11 ± 0.07	[illegible]
W.L. 289	not 1, or 2	2.25 ± 0.03	2.22 ± 0.06	[illegible]
W.L. 296	not 1, or 2	2.42 ± 0.08	2.55 ± 0.07	[illegible]
W.L. 300	not 1, or 2	2.30 ± 0.14	2.36 ± 0.06	[illegible]
W.L. 311	not 1, or 2	1.82 ± 0.06	1.82 ± 0.05	[illegible]
W.L. 44	1, not 2	1.53 ± 0.06	1.87 ± 0.05	[illegible]
W.L. 55	1, not 2	1.53 ± 0.05	1.96 ± 0.03	[illegible]
W.L. 241	1, not 2	1.62 ± 0.08	2.34 ± 0.04	[illegible]
W.L. 274	1, not 2	1.71 ± 0.10	2.34 ± 0.06	[illegible]
$B^{1/1}$	1	1.53 ± 0.04	1.89 ± 0.04	
$B^{2/2}$	2	1.90 ± 0.10	1.30 ± 0.06	

* See Table 10 Results expressed as mean log spleen weight ± s.e. Hence $\bar{X}2$ [illegible] ratio of mean spleen weight in $B^{2/2}$ embryos/mean spleen weight in $B^{1/1}$ embryos [illegible]

data validating the typing. Table 11 shows some additional data from four W.L. donors which were pre-typed as being AA1, and nine others found not to be AA1. There have been no failures of the [illegible] photographic view of [illegible] compatibility test. W[illegible] should react in B[illegible]

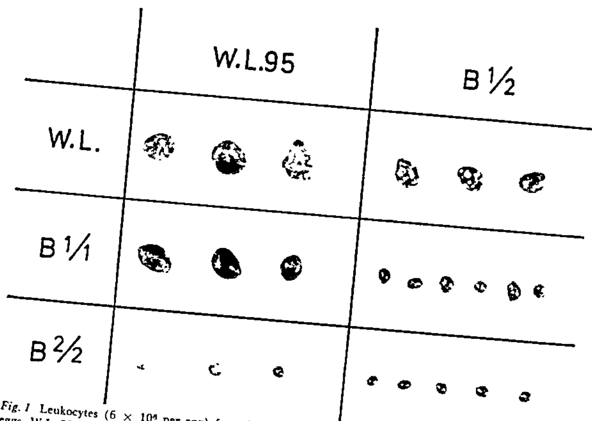


Fig. 1 Leukocytes (6 × 10⁶ per egg) from 2 donors were injected into groups of $B^{1/1}$, $B^{2/2}$ and W.L. eggs. W.L. 95 was typed as AA2-positive.

not in $B^{2/2}$. Control $B^{1/2}$ leukocytes should of course react in W.L. only.

DISCUSSION

The vast literature which existed on histocompatibility typing in mouse and man was already most impressive in respect of the serological definition of particular loci which happened to be the easiest to demonstrate with the serological methods employed, before it was realised that the probably most important antigens of the MHS passed undetected.

The present work with chickens drew a lesson from that development, and proceeded to eliminate by absorption with blood from unrelated birds as many demonstrable antibodies as possible so long as they failed to inhibit allo-aggression. Nevertheless, the absorptions guided by the GVH reaction as described in Results, Section 2, always left a serum which retained the dual properties of haemagglutinating both related and unrelated birds which carried the same allo-aggression antigen(s) as the original donor for the serum production, and to inhibit the T cells of the same individuals in their own GVH reactivity. Such sera could then be used for the typing of new unrelated birds. The validity of the typing was finally demonstrated *in vivo* by the absence of allo-aggression when WBC from properly matched outbred donors were injected into $B^{1/1}$ and $B^{2/2}$ embryos.

Thus the AA antigens seem alone to determine whether or not the direct confrontation *in vivo* of allogeneic lymphocytes will result in a severe reaction, measured in these experiments by GVH splenomegaly. However, there is also a just visible, small spleen enlargement, and the occasional yellowish pock to be found in the injected spleens in spite of AA matching. But these changes are hard-

ly more pronounced than what is found also by the injection of WBC within B-group syngeneic inbreds, as illustrated by Fig. 1. Nevertheless, though feeble, such changes are probably also due to histoincompatibility, traditionally ascribed to "minor" loci outside the MHS; such loci are poorly defined as yet in chickens.

The point I want to raise in this context is the possibility that there may also be minor histocompatibility loci which form part of the B-system. This possibility seems to be the most reasonable explanation of the situation encountered in Table 10, where the anti-B1 serum inhibited the GVH reactivity not only of AA1-positive but also of AA1-negative cells. If this interpretation is correct, it clearly means that the simple inhibition of GVH reactivity used in Table 10 is not as discriminatory a test as is the indirect test based on absorption of GVH inhibitory antibody shown in Table 3, and used for further screening in Table 4. It is only the latter test which permits effective matching for the AA locus. (The possibility that the AA "locus" itself may eventually prove to be composed of several linked loci can of course not be dismissed).

It is an interesting and important point whether the antibodies in an AA typing serum which cause the agglutination of erythrocytes of the right phenotype are of the very same specificity as those which inhibit the GVH reactions of the T cells of the same individuals. So far, we can be certain that RBC alone can remove the inhibitory antibodies, and that the injection of WBC alone leads both to the formation of inhibitory antibodies and to AA haemagglutinins (*Crone & Simonsen*, to be published). We favour the hypothesis that the same specificities are involved, and have so far no factual evidence which suggests that they are different.

Also the Ir (immune response) genes are currently under separate study in these birds. There is a clear-cut difference in the antibody response to TGAL between the $B^{1/1}$ and $B^{4/4}$ lines which follows the AA antigens in family studies but do not appear to follow the corresponding AA antigens in studies of outbred W.L.s (*Koch & Simonsen*, to be published).

The apparent identity of the B1 haplotype in the inbred $B^{1/1}$ and in outbred W.L.s (Table 8) is noteworthy, even though it may be restricted to that part of the B1 haplotype which is different from B2. The possibility that they may differ in respect of determinants which have been hidden in the present experiments because they are shared by the B2 haplotype should certainly be kept in mind. But such "hidden determinants" are at least most unlikely to represent an additional allo-aggression locus. If they did, we should not expect the good match actually found by the direct confrontation of AA1-positive W.L. donor cells and $B^{1/1}$ recipients (Table 11).

Let us assume from the available evidence that the B1 haplotype is nearly identical in the inbred $B^{1/1}$ birds of Iowa and the two outbred W.L. flocks so far studied in Denmark. If so, this haplotype appears to be quite common, although I admittedly do not know the size of the founding stocks of the flocks. If we exclude from Table 4 the experiments where the GVH-typing was done in groups which were pre-selected on basis of sero-typing, and consider those groups only where all members of a new panel were typed by the GVH method, we can arrive at estimates for the gene frequency of the AA1 gene in the two flocks. Using the standard formula of $p = 1-\sqrt{1-F}$, where F is the observed AA1 phenotype, we will arrive at the values of $p = 0.07$ in flock I ($N = 15$), and $p = 0.10$ in flock II ($N = 87$).

The negative results from all attempts to show a difference between the inbred B1 haplotype and the haplotype(s) in outbred AA1-positive W.L.s may thus be accounted for by the assumption that there simply is no difference. If so, one would normally assume a selective advantage for the linkage disequilibrium by which the AA1 antigens have been kept associated with all the rest of the haemagglutinogens in the B1 haplotype. Admittedly, we do not know at all how

many these other antigens are, and yet the extent of cross-reaction found within unrelated birds (Table 1) makes it tempting to guess that the number of linked loci for haemagglutinogens in the B-system will be considerably more than the 2–3 serological defined loci to which the H-2 and HL-A registers seem confined.

Alternatively, there may be no essential difference between the B-system and the homologous mammalian systems. The present apparent differences could all be explained by a more complete expression of the gene products of the MHS in the nucleated RBC of the birds.

This work has been supported by the *Danish Medical Research Council.*

REFERENCES

Atkins, R. C. & Ford, W. L.: Early cellular events in a systemic graft-versus-host reaction. 1975, in press.

Bach, F. H., Bock, H., Graupner, K., Day, E. & Klostermann, H.: Cell kinetic studies in mixed leukocyte cultures: An in vitro model of homograft reactivity. Proc. Nat. Acad. Sci. Wash. *62:* 377–384, 1969.

Briles, W. E., McGibbon, W. H. & Irwin, M. R.: On multiple alleles effecting cellular antigens in the chicken. Genetics *35:* 633–652, 1950.

Crone, Monna, Koch, C. & Simonsen, M.: The elusive T cell receptor. Transplant. Rev. *10:* 36–56, 1972.

Ford, W. L & Simonsen, M.: The factor of immunization in the rat. J. Exp. Med. *133:* 938–949, 1971

Hildemann, W H · Phylogeny of immune responsiveness in invertebrates. Life Sciences *44:* 605–614, 1974

Jaffe, W P. & McDermid, E. M.: Blood groups and splenomegaly in chick embryos. Science *137:* 984–984, 1962

Jerne, N. K The somatic generation of immune recognition Eur J Immunol. *1:* 1–9, 1971.

Katz, D. H., Hamaoka T & Benacerraf, B.: Cell interaction between histoincompatible T and B lymphocytes II Failure of physiologic cooperative interaction between T and B lymphocytes from allogeneic donor strains in humoral response to hapten-protein conjugates. J. Exp. Med. *137:* 1405–1418, 1973.

Lilly, F., Boyse, E. A. & Old, L. J.: Genetic basis of susceptibility to viral leukaemogenesis. Lancet *2:* 1207–1209, 1964.

McBride, R. A., Coppleson, L. W., Nisbet, N. W., Simonsen, M., Skowron-Cendrzak, Anna, & Wigzell, H. L. R.: Accelerated maturation in the chick. Immunology *10:* 63–79, 1966.

McDevitt, H. O. & Landy, M. (eds.): Genetic control of immune responsiveness. Relationship to disease susceptibility. Academic Press. New York, 1972.

Nielsen, H. E.: Reactivity of lymphocytes from germfree rats in mixed leukocyte culture and in graft-versus-host reaction. J. Exp. Med. *136:* 417–425, 1972.

Schierman, L. W. & Nordskog, A. W.: Influence of the B blood group-histocompatibility locus in chickens on a graft-versus-host reaction. Nature *197:* 511–512, 1963.

Shevach, E. M. & Rosenthal, A. S.: Function of macrophages in antigen recognition by guinea pig lymphocytes. J. Exp. Med. *138:* 1213–1229, 1973.

Simonsen, M.: The factor of immunization: Clonal selection theory investigated by spleen assays of graft-versus-host reaction. In: Wolstenholme, G. E. W. & Cameron, M. P. (Eds.). Transplantation. J. & A. Churchill, London 1962, pp. 182–215.

Simonsen, M.: The clonal selection hypothesis evaluated by grafted cells reacting against their hosts. Cold Spring Harbor Symp. Quant. Biol. *32:* 517–523, 1967.

Simonsen, M.: Tissue typing: An experimental solution in the chicken. J. Immunol. *111:* 1607–1609, 1973.

Svejgaard, A., Platz, P., Ryder, L. P., Nielsen, L. S. & Thomsen, M.: HL-A and disease associations.—A survey. Transplant. Rev. 1975, in press.

Todd, C.: Cellular individuality in the higher animals, with special reference to the individuality of the red blood corpuscle Proc. Roy Soc. (London) *106:* 20–44, 1930.

Widmer, M. B., Sandel, P. M. & Bach, F H.: Responsiveness of germfree mice in mixed leukocyte culture. J. Exp. Med. *136:* 398–402, 1972.

Wilson, D. B., Blyth, J L. & Nowell, P C.: Quantitative studies in the mixed lymphocyte interaction in rats. J. Exp. Med. *128·* 1157–1181, 1968.

Acta path microbiol. scand. Sect. C, 83, 15–18, 1975

DELAYED HYPERSENSITIVITY TO STAPHYLOCOCCAL PROTEIN A DETECTED BY LEUCOCYTE MIGRATION INHIBITION

SVEIN M. HELGELAND, RAGNAR R. NÆSS and ARNE GROV

The University of Bergen, School of Medicine, The Gade Institute, Department of Microbiology, Bergen, Norway

Helgeland, S. M., Næss, R. R. & Grov, A. Delayed hypersensitivity to staphylococcal protein A detected by leucocyte migration inhibition. Acta path. microbiol. scand Sect. C, 83: 15–18, 1975.

Delayed hypersensitivity to staphylococcal protein A was indicated by the migration inhibition reaction (MIR), using peritoneal cells from protein A sensitized guinea pigs. No cross-reactivity between protein A, polysaccharide A and peptidoglycan was found. Peptidoglycan from *Staphylococcus aureus* strain Cowan I inhibited almost completely the migration of cells from both sensitized and unsensitized cells.

Key words: Staphylococcal protein A; delayed hypersensitivity; leucocyte migration inhibition.

Helgeland, S. M., Mikrobiologisk avdeling, MFH-bygget, N-5016 Haukeland sykehus, Norway.

Received 8.vii.74 Accepted 12.vii.74

Antigens of *Staphylococcus aureus* have been shown to elicit cell-mediated immunity which seems to be of significance in the pathogenesis of staphylococcal infections (1, 9, 10, 11, 13, 14, 15). *Kowalski* & *Berman* (11) found a more protracted indurated inflammatory phase of the dermal reaction in animals sensitized with cell wall and cell wall components than in unsensitized animals. Since the reactions to cell walls and peptidoglycan complexes did not differ in size and gross morphology, it was proposed that the peptidoglycan complex is responsible for both the acute inflammatory lesions and hypersensitivity reactions. However, later studies including germ-free animals and the observation that a migration inhibition factor could not be detected in supernatants of cell cultures incubated with peptidoglycan, suggested that the reaction to the peptidoglycan complex of cell walls is due to an intrinsic toxic factor and not an immunologic mechanism (6, 7).

Delayed hypersensitivity to staphylococcal cell walls has, however, been shown to be passively transferred by peritoneal exudate cells from sensitized guinea pigs (12). This was also the result obtained by *Heczko et al.* (8) using the staphylococcal cell wall antigen protein A. Delayed hypersensitivity to this antigen was passively transferred by lymphoid cells but not by immunsera.

The purpose of this study was to reexamine the action of protein A and other defined cell wall antigens from staphylococci in the migration inhibition test.

MATERIALS AND METHODS

Antigens

Protein A was isolated from *Staphylococcus aureus* strain Cowan I according to the method described by *Grov* (2). Polysaccharide A (poly A) (β-N-acetyl-glucosaminylribitol teichoic acid) from *Staphylococcus aureus* strain Wood 46 was isolated and purified according to the method described by *Haukenes* (4). Peptidoglycan from *Staphylococcus aureus* strains Wood 46 and Cowan I was prepared and examined for purity as previously described (5).

Sensitization of Animals

Groups consisting of 4 guinea pigs (inbred albino guinea pigs weighing 350–400 g of both sexes) were immunized as follows:

One group received protein A (100 µg/0.25 ml of saline) emulsified in an equal volume of complete Freund's adjuvant (FCA) (Difco), a second group poly A (200 µg/0.25 ml of saline) in FCA, and a third group peptidoglycan from strain Wood 46 (200 µg/0.25 ml of saline) in FCA.

Each guinea pig received an immunizing volume of 0.5 ml, distributed equally in both hind foot pads.

Migration Inhibition Test

Guinea pig peritoneal exudate cells were employed. Ten days after sensitization with antigen the guinea pigs were injected intraperitoneally with 20 ml sterile paraffin oil (Merck art. 7160) to induce exudate. Five days later the animals were killed and bled out by cutting the neck vessels. The peritoneal exudate was collected in Hank's BSS with heparin (15 I.U./ml). Cells from sensitized animals were pooled to avoid possible individual differences in reactivity. The cells were washed three times in chilled Hanks' BSS with heparin by centrifugation for 5 min at 1000 rev/min, and then once in Eagle's medium (GIBCO, Grand Island, N.Y. USA) containing 100 I.U./ml penicillin (Apothekernes Lab., Oslo), 100 µg/ml of streptomycin (Glaxo Labs., Greenford, England); 10 µg/ml of auromycin (Lederle Lab. Div., Pearl Riv., N.Y. USA), and 10 per cent heat inactivated calf serum. The concentration of cells was adjusted to about $2 \cdot 10^7$ cells/ml of the supplemented Eagle's medium. Differential count of the cell suspension revealed approximately 60–70 per cent macrophages and 30–40 per cent lymphocytes. The cells were transferred by means of vacuum suction to heparinized microhematocrit capillary tubes (capacity approx. 75 µl) (Vitrex, Denmark) which had prior to filling been sealed at one end by melting the glass. The cells harvested from one animal were sufficient to fill 10–15 capillary tubes. The capillary tubes were centrifuged at 1000 rev/min for 10 min and cut just below the cell-fluid interphase. The portion containing the cells was immediately placed in small petri dishes (4.5 cm in diameter) (Sterilin Ltd., England), which contained 4 ml of supplemented Eagle's medium. Three capillaries were held in place in each chamber by a small amount of silicone (Dow Corning Corp.). For each of the sensitized cell preparations four chambers with antigen and four chambers without antigen were prepared. To make sure that the antigens had no toxic effect, antigens in the same concentrations as those used in test chambers were added to the culture medium in chambers with unsensitized cells. Antigen concentrations varying from 12.5 µg/ml to 150 µg/ml were tested in each of the cell systems. The cell migration was examined in an inverse microscope and photographed with a Polaroid camera. The area of migration was calculated by planimetry. The following formula was used to express the extent of migration of exudate cells:

$$\frac{\text{Average area of migration with antigen}}{\text{Average area of migration without antigen}} \times 100 =$$

percentage migration with antigen.

At least 12 capillary tubes were used for calculation of average area.

RESULTS

The results which are summarized in Table I show that the migration of peritoneal cells from animals sensitized with protein A was markedly inhibited by 50 µg of protein A/ml when examined after 18 h. An almost identical effect on sensitized cells was seen using 25 µg/ml protein A (Fig. 1). In concentrations up to 150 µg/ml protein A no effect was observed on normal unsensitized cells. Protein A gave no inhibition of cells from animals injected with poly A or peptidoglycan. Poly A gave a weak inhibition of cells from all groups of animals. The significance of the 80 per cent inhibition with protein A on cells from protein A sensitized animals was supported by the fact that protein A, for some unknown reason, seemed to stimulate the migration of cells which had not been sensitized with protein A. Peptidoglycan from strain Wood 46, which does not contain protein A, gave more than 80 per cent migration inhibition of both sensitized and non-sensitized cells

TABLE 1. *Mean Cell Migration (per cent)*

Cells from animals injected with	Antigens (50 µg/ml) in medium			
	protein A	poly A	peptidoglycan	control
protein A	22*	68	<20	100
poly A	114	80	<20	100
peptidoglycan	115	85	<20	100
no antigen	105	84	<20	100

* The difference between this group and the control group was statistically significant ($t=3.08$; $p<0.01$).

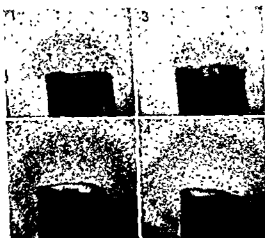


Fig. 1. Migration of sensitized and unsensitized cells in the presence of different antigens.

1. cells sensitized with protein A, 50 µg protein A/ml medium.
2. unsensitized cells, 25 µg protein A/ml medium.
3. unsensitized cells, 12.5 µg peptidoglycan/ml medium.
4. unsensitized cells, 50 µg poly A/ml medium.

at a concentration of 50 µg per ml. Marked inhibition was also observed at a concentratio of 12.5 µg/ml.

DISCUSSION

The use of the in vitro technique, migration inhibition test (MIT) has supported the finding of *Heczko et al.* (8), that protein A elicits a delayed hypersensitivity reaction. We were not able to demonstrate any crossreactivity between protein A, peptidoglycan and the polysaccharide A, although all have peptidoglycan fragments in common (3). The ultrasonically treated peptidoglycan strongly inhibited the migration of all cells even in as low concentration as 12.5 µg/ml. This inhibition, which has also been found with peptidoglycan from other bacterial species has been interpreted as being due to a toxic effect (6). The migration of cells from animals injected with poly A did not differ significantly from those from unsensitized animals in the presence of poly A. This indicates, as also found by *Heczko et al.* (unpublished data), that poly A does not elicit delayed hypersensitivity. The weak inhibition of unsensitized cells in the presence of poly A may be due to the peptidoglycan fragment, which contains more determinants than that of protein A (3). The stimulating effect of protein A on cells not sensitized with photein A remains unclear and should be further investigated.

REFERENCES

1. *Bõe, J.:* Investigations on the importance of bacterial allergy for the development of cutaneous infections due to staphylococci. Acta Dermatol. Venereol. *26:* 111–136, 1946.
2. *Grov, A.:* Studies on antigen preparations from *Staphylococcus aureus*. 4. Separation and purification of protein A and a related precipitinogen. Acta path. microbiol. scand. *73:* 400–406, 1968.
3. *Grov, A.:* Studies on antigen preparations from *Staphylococcus aureus*. 8. The substance of crude protein A sensitizing tanned erythrocytes. Acta path. microbiol. scand. *76:* 629–636, 1969.
4. *Haukenes, G.:* Immunochemical studies on polysaccharide A of *Staphylococcus aureus*. 2. Further studies on purification methods. Acta path. microbiol. scand. *55:* 117–126, 1962.

5. *Helgeland, S. M. & Grov, A.:* Immunochemical characterization of staphylococcal and micrococcal mucopeptides. Acta path. microbiol. scand. *79:* 819–826, 1971.
6. *Heymer, B., Bültmann, B. & Haferkamp, O.:* Toxicity of streptococcal mucopeptides *in vivo* and *in vitro*. J. Immunol. *106:* 858–861, 1971.
7. *Heymer, B., Bültmann, B., Schachenmayr, W., Spanel, R., Haferkamp, O. & Schmidt, W. C.:* Migration inhibition of rat peritoneal cells induced by streptococcal mucopeptides. Characteristics of the reaction and properties of the mucopeptide preparations. J. Immunol. *116:* 1743–1754, 1973.
8. *Heczko, P. B., Grov, A. & Oeding, P.:* *In vivo* reactions of staphylococcal antigens. 1. Hypersensitivity to protein A. Acta path. microbiol. scand. Sect. B, *81: 731–740, 1973.*
9. *Johanovsky, J.:* Role of hypersensitivity in experimental staphylococcal infection. Nature (Lond.) *182:* 1454–1456, 1958.
10. *Johnson, J. E., III, Cluff, L. E. & Goshi, K.:* Studies on the pathogenesis of Staphylococcus infections. I. The effect of repeated skin infection. J. Exp. Med. *113:* 235–247, 1961.
11. *Kowalski, J. J. & Berman, D. T.:* Immunological activity of cell wall antigens of *Staphylococcus aureus*. Infect. Immunity *4:* 205–211, 1971.
12. *Targowski, S. P. & Berman, D. T.:* Passive transfer of delayed-type hypersensitivity to cell walls of *Staphylococcus aureus*. Infect. Immunity *9: 41–43, 1974.*
13. *Taubler, J. H.:* Staphylococcal delayed hypersensitivity in mice. I. Induction and *in vivo* demonstration of delayed hypersensitivity. J. Immunol. *101:* 546–549, 1968.
14. *Taubler, J. H., Grieb, M. & Mudd, S.:* Immunologically induced and elicited local resistance to *Staphylococcus aureus*. Infect. Immunity *2: 757–761, 1970.*
15. *Taubler, J. H. & Mudd, S.:* Staphylococcal delayed hypersensitivity in mice. II. *In vitro* demonstration and specificity of delayed hypersensitivity. J. Immunol. *101:* 550–555, 1968.

Acta path. microbiol scand. Sect. C, *83:* 19–27, 1975

SMOOTH-MUSCLE ANTIBODIES IN SERA WITH PAUL-BUNNELL HETEROPHIL ANTIBODIES

Paul Andersen

Institute of Medical Microbiology, University of Aarhus, Aarhus, Denmark

Andersen, P. Smooth-muscle antibodies in sera with Paul-Bunnell heterophil antibodies. Acta path. microbiol. scand. Sect. C, *83:* 19–27, 1975

A total of 115 Paul-Bunnell-positive and 40 Paul-Bunnell-negative sera were studied for smooth-muscle antibodies (SMA) and some other tissue antibodies by means of the indirect immunofluorescent method. SMA were found in 56 per cent of the Paul-Bunnell-positive sera and in 23 per cent of the negative sera ($0.001 > p$). The SMA were mainly of the IgM class, but antibodies of the IgG and the IgA class were also demonstrated. SMA of the IgM class were found most frequently in sera with Paul- Bunnell antibodies in high titres. This relationship could not be demonstrated for SMA of the IgG or the IgA class. In a few persons, two or three serum samples were obtained, and it was found that the IgM-SMA disappeared within a few weeks, while the Paul-Bunnell antibody titre remained virtually unchanged. All SMA-positive sera reacted with rat gastric smooth-muscle fibres and rat renal vessel walls in the same or approximately the same titres. In addition, some of the positive sera also reacted with rat renal glomeruli, which suggests that antibodies with different specificity may occur. Tissue antibodies other than SMA were rare, occurring with the same frequency in the Paul-Bunnell-positive and -negative sera.

Key words: Smooth-muscle antibodies; Paul-Bunnell antibodies.

P. Andersen, Institute of Medical Microbiology, Bartholin Building, University of Aarhus, DK-8000 Aarhus C, Denmark.

Received 20.vii.74 Accepted 20.vii.74

Smooth-muscle antibodies (SMA) have been demonstrated by means of the indirect immunofluorescent method in sera from patients with lupoid hepatitis (8), viral hepatitis (1, 15), certain malignant diseases (13) and infectious mononucleosis (7). Sera with SMA react with stomach smooth muscle and blood-vessel walls. Many of these sera also react with renal glomeruli (9, 14), which suggests that antibodies with different specificity may occur.

The occurrence of SMA and some other tissue antibodies in Paul-Bunnell-positive sera and the correlation between SMA and Paul-Bunnell heterophil antibodies were investigated. In order to determine the organ specificity of the antibodies, the reactivity of SMA with different antigens present in rat tissue was also studied.

MATERIALS AND METHODS

Sera

A total of 155 sera from 140 persons were selected for investigation. The sera had been sent to Statens Seruminstitut in Copenhagen to be examined for Paul-Bunnell heterophil antibodies. The

2*

sera were divided into two groups according to the Paul-Bunnell reaction.

Group 1. 40 sera with a negative Paul-Bunnell reaction, i.e. titre below 8. They were obtained from 21 females and 19 males. The age range of this group was from 6 months to 57 years.

Group 2. 115 sera with a positive Paul-Bunnell reaction with titres from 64 to 2048. From each of 38 women and 51 men only one serum sample was obtained, while two or three samples were obtained from eight women and three men. All sera from each of the latter patients were studied for SMA in the same experiment. The age range of this group was from 6 to 42 years.

Methods

Paul-Bunnell heterophil antibodies were demonstrated as described by *Kristensen* (11).

Smooth-muscle antibodies (SMA), antinuclear antibodies (ANA), mitochondrial antibodies (MTA) and parietal cell antibodies (PA) were demonstrated by the indirect immunofluorescent method (IIF) as described earlier (2). The substrates were sections of rat stomach and rat kidney, 4 mu in thickness. For SMA, the staining reactions of smooth-muscle fibres of the rat stomach, the vessel walls in the kidney and components of the renal glomeruli were recorded. ANA were demonstrated by their reactivity with the nuclei of tubular cells of the kidney. MTA stained the cytoplasm of the renal tubular cells and the gastric parietal cells, while PA yielded staining of only the gastric parietal cells. All sera were examined with three fluorescein (FITC) conjugated anti-human immunoglobulins (Ig). A goat anti-human IgG conjugate with a molar F:P ratio of 4.2 and an antibody concentration of 3.4 mg per ml was obtained from Professor *E. H. Beutner*, State University of New York, Buffalo, USA. A sheep anti-human IgA conjugate with a molar F:P ratio of 4.1 and a sheep anti-human IgM conjugate with a molar F:P ratio of 3.0 were supplied by Wellcome Research Laboratories, England. The antibody content of the latter conjugates were 4 units per ml for both the anti-IgA and the anti-IgM conjugate (4). Conjugates and sera were diluted in phosphate-buffered saline (phosphate, 0.118 mol; NaCl, 0.111 mol; pH 7.2) with 2 per cent bovine serum albumin. The sera were tested in a dilution of 1:10 and all positive or doubtfully positive sera were titrated in doubling dilutions, starting with 1:10. The stained sections were examined under a fluorescence microscope with interference filters as described previously (3).

Absorption Experiments

Six sera with Paul-Bunnell antibodies and SMA of the IgM class were absorbed with a lyophilized tissue extract prepared from human myometrium, referred to as crude actomyosin, as described earlier (3). The same sera were also absorbed with sheep red-blood cells (SRBC). The sera were diluted 1:5 with 50 per cent SRBC, and the absorption was carried out at room temperature for one hour. After absorption the sera were centrifuged and the supernatants were examined for SMA by the IIF and for agglutinins to SRBC.

RESULTS

SMA which react with rat gastric smooth muscle (RS) were demonstrated with a higher frequency in Paul-Bunnell-positive sera (56 per cent) than in Paul-Bunnell-negative sera (23 per cent); this difference was statistically significant by the chi-square test ($0.001 > p > 0.0005$). The antibodies were of the IgG, the IgA as well as the IgM class. It is seen from Table 1 that antibodies of the

TABLE 1. *The Occurrence of Smooth-Muscle Antibodies in Paul-Bunnell-Positive and Paul-Bunnell-Negative Sera*

	No. of sera	No. of sera with smooth-muscle antibodies			
		IgG, IgA or IgM antibodies	IgG antibodies	IgA antibodies	IgM antibodies
Paul-Bunnell-negative	40	9 (23%)	5 (13%)	0 (0%)	6 (15%)
Paul-Bunnell-positive	115	64 (56%)	29 (25%)	5 (4%)	49 (43%)
		$0.001 > p > 0.0005$*	$0.20 > p > 0.10$*	$p \approx 0.22$**	$0.01 > p > 0.01$*

* chi-square test; ** Fisher's extract test.

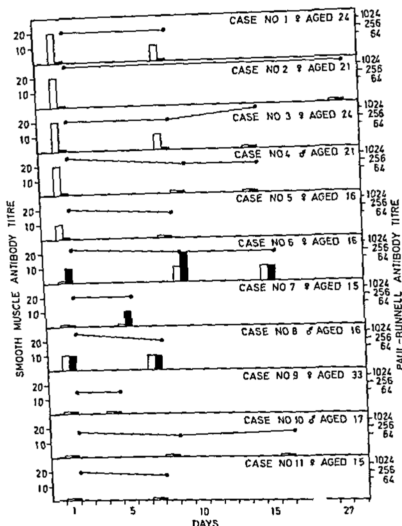


Fig. 1. Smooth-muscle antibody titres and Paul-Bunnell antibody titres in 26 sera from 11 persons. The white bars represent the titres of the IgM smooth-muscle antibodies and the black bars represent the titres of the IgG smooth-muscle antibodies. The dots represent the Paul-Bunnell antibody titres.

IgM class occurred in 43 per cent of the Paul-Bunnell-positive sera and in 15 per cent of the Paul-Bunnell-negative sera. This difference was found to be statistically significant by the chi-square test ($0.01 > p > 0.001$). SMA of the IgG and the IgA class were also found to occur more frequently in the Paul-Bunnell-positive sera than in the Paul-Bunnell-negative sera, but these differences were not significant.

Fig. 1 shows the SMA titres and the Paul-Bunnell titres in 26 sera from 11 persons from whom two or three serum samples were obtained. In five persons (Nos. 1–5), the IgM-SMA titre decreased in one to four weeks, and in one (No. 6) the IgM-SMA titre increased from below 10 to 10. The Paul-Bunnell titres remained virtually unchanged. SMA of the IgG class were found in three persons (Nos. 6–8). In two of these, the antibody titre increased, while the titre remained unchanged during the observation period in the third. In seven sera from three persons (Nos. 9–11), SMA could not be de-

monstrated. In these sera, the Paul-Bunnell *antibody titres* were between 64 and 256, while the titres ranged from 256 to 2048 in *sera with* SMA.

Paul-Bunnell Titres and the Occurrence of SMA

The relationship between the level of the Paul-Bunnell antibody titre and the number of sera with SMA was studied for each of the three Ig classes separately, and the results are shown in Fig. 2. Because of the low number of sera with Paul-Bunnell titres above 256 (26 sera), sera with titres of 256 and 512 (47 sera) were pooled in one group, and so were sera with titres of 1024 and 2048 (17 sera). It is seen that SMA of the IgM class were found in 15 per cent of the Paul-Bunnell-*negative sera and in 12 per cent of the sera* with Paul-Bunnell antibodies in a titre of 64. At higher Paul-Bunnell titres, an increase in the percentage of sera with SMA occurred. As regards antibodies of the IgG and the IgA class, the increase of samples with SMA was less pronounced, i.e. from 13 per cent to 24 per cent and from 0 per cent to 6 per cent, respectively. The correlation between the Paul-Bunnell antibody titre and the percentage of samples with SMA was examined by a *rank correlation test. It was found to be statistically* significant for SMA of the IgM class ($0.0001 > p$), but not significant for SMA of the IgG and IgA classes ($p = 0.066$ and $p = 0.067$, respectively).

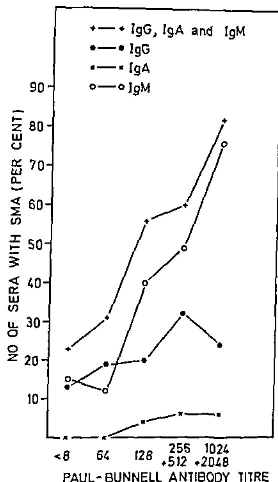


Fig 2. The relationship between the Paul-Bunnell *antibody titre* and the number of sera with smooth-muscle antibodies.

Relationship between the SMA and the Paul-Bunnell Titres

The relationship between the IgM-SMA *titres* and the Paul-Bunnell *antibody titres* was also studied. It is seen from Table 2 that SMA were found in sera without Paul-Bunnell antibodies, and that Paul-Bunnell antibodies were found in titres between 64 and 2048 in sera without SMA. However, it also appears from Table 2 that sera with Paul-Bunnell antibodies in high titres also *often contained* SMA *in high titres*. This relationship was examined by a Spearman's *rank correlation* test with correction for identical ranks, and it was found to be statistically significant ($r_s = 0.267$, $0.01 > p > 0.001$). Sera which did not contain either SMA or Paul-Bunnell antibodies were excluded from this analysis.

Absorption Experiments

After absorption of six sera with crude actomyosin the reactivity with gastric smooth muscle and renal glomeruli was abolished, while *the* reactivity with SRBC was unaltered. Thus, the antibodies reacting with both gastric smooth muscle and renal glomeruli could be absorbed with the same tissue preparation. Absorption of the same sera with

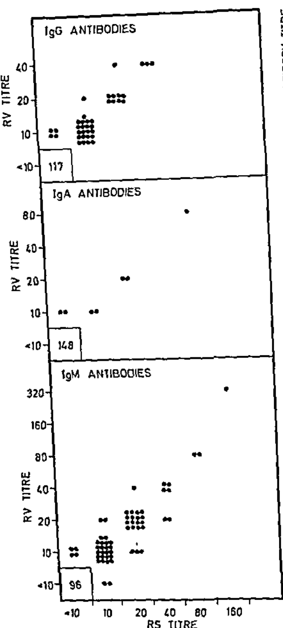


Fig 3. Smooth-muscle antibody titres of 155 sera titrated with rat stomach smooth muscle as antigen (RS titre) and with rat vessel walls as antigen (RV titre).

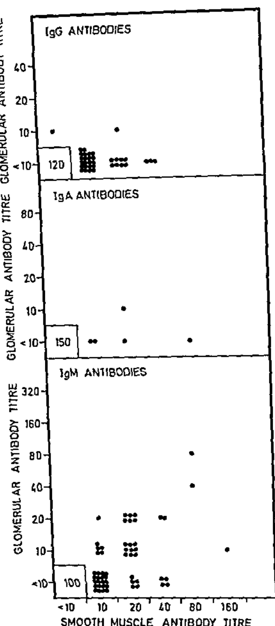


Fig. 4. The relationship between the smooth-muscle antibody titre (antigen: stomach smooth muscle) and the glomerular antibody titre in 155 sera.

SRBC did not alter the reactivity with gastric smooth muscle or renal glomeruli, while the agglutination of SRBC was abolished. Thus, the Paul-Bunnell antibodies and the SMA were not cross-reacting.

Specificity of SMA

The titres of SMA reacting with rat stomach smooth muscle (RS titre) and with rat vessel walls (RV titre) were compared (Fig. 3). In most cases, the two titres were

TABLE 2. *Paul-Bunnell Antibody Titres and IgM-Smooth-Muscle Antibody Titres in 155 Sera*

Paul-Bunnell antibody titre	Smooth-muscle antibody titre < 10	10	20	40	80	160
< 8	34	5	0	0	1	0
64	23	3	0	0	0	0
128	15	0	6	3	1	0
256	20	9	5	3	0	1
512	4	3	2	0	0	0
1024	1	6	3	0	0	0
2048	3	0	4	0	0	0

identical, and the difference never exceeded one serum dilution. For each of the Ig classes, the relationship between the RS titres and the RV titres was found to be statistically significant by a Spearman's rank correlation test with correction for identical ranks. Sera in which both titres were below 10 were not included in the analysis (IgG antibodies r_s = 0.3760, 0.025 > p > 0.020;, IgA antibodies r_s = 0.9221, 0.01 > p; IgM antibodies r_s = 0.8417, 0.001 > p).

Some sera also contained antibodies which reacted with rat renal glomeruli, here referred to as glomerular antibodies. With one exception, all these sera also reacted with gastric smooth muscle. On the other hand, sera could react with gastric smooth muscle, even in high titres, without yielding staining with glomeruli (Fig. 4). As regards IgG antibodies, only two sera reacted with renal glomeruli, while 35 reacted with stomach smooth muscle. SMA of the IgA class were found in five sera, but only one of these did also react with renal glomeruli. As regards SMA of the IgM class, a relationship between the SMA titres and the glomerular antibody titre was demonstrated. This relationship was found to be statistically significant by a Spearman's rank correlation test with correction for identical ranks (r_s = 0.4767, 0.001 > p). How-

TABLE 3. *Tissue Antibody Titres and Paul-Bunnell Antibody Titres in 10 Sera from Five Women (F) and Two Men (M)*

Patient		P-B	SMA		ANA		PA	MTA
Sex	Age	titre	IgG	IgM	IgG	IgM	IgG	IgG
F	24	256	–	10	–	–	10	–
		512	10	–	–	–	10	–
F	16	256	20	–	–	–	10	–
		256	10	–	–	–	10	–
F	33	64	–	–	–	40	–	–
		64	–	–	–	40	–	–
M	21	256	20	40	–	20	–	–
F	10	256	–	10	–	10	–	–
F	½	< 8	–	10	10	–	–	–
M	10	< 8	–	–	–	–	–	10

P-B, Paul-Bunnell antibody; SMA, smooth-muscle antibody; ANA, antinuclear antibody; MTA, mitochondrial antibody; PA, parietal-cell antibody; –, titre below 10.



Fig. 5. Indirect immunofluorescent staining of a rat kidney section with IgM antibodies of serum 1926. Staining of a glomerulus and the walls of a blood vessel is seen.



Fig 6. Indirect immunofluorescent staining of a rat kidney with IgA antibodies of serum 1926. Staining of the walls of a vessel is seen, while a glomerulus beside the vessel is unstained.

ever, the SMA titres were generally higher than the glomerular antibody titres. One serum contained IgM-SMA in a titre of 160 and glomerular antibodies in a titre of 10, and nine sera with SMA in titres of 20 and 40 did not contain glomerular antibodies Another serum with IgM antibodies which reacted with gastric smooth muscle and renal glomeruli in a titre of 20 also contained IgA smooth-muscle antibodies. The IgA antibodies reacted with gastric smooth muscle and vessel walls in a titre of 80, but did not react with renal glomeruli. The reactivity of this serum with kidney sections is shown in Figs. 5 and 6.

Other Tissue Antibodies

Antinuclear antibodies (ANA), parietal cell antibodies (PA) or mitochondrial antibodies (MTA) were found in sera from five of the Paul-Bunnell-positive and in two of the Paul-Bunnell-negative persons. The results are shown in Table 3. The incidence of these antibodies was low, and no relationship between the concurrent occurrence of SMA or the level of the Paul-Bunnell antibody titre could be established. However, it should be mentioned that the ANA found in sera with Paul-Bunnell antibodies were all of the IgM class.

DISCUSSION

SMA were found more often in Paul-Bunnell-positive than in Paul-Bunnell-negative sera, and they were predominantly of the IgM class, although antibodies of the IgG and the IgA class were also demonstrated. Absorption experiments revealed that SMA of the IgM class and Paul-Bunnell heterophil antibodies were not cross-reacting. However, a simultaneous occurrence of these two antibodies in sera was often observed. In the few cases in which two or three serum samples were available, it was found that SMA of the IgM class occurred in the highest titres in the first serum samples and then disappeared within a few weeks, while the Paul-Bunnell antibody titre remained virtually unchanged. Other tissue antibodies occurred only rarely and with the same frequency in Paul-Bunnell-positive and -negative sera. This is in agreement with some previous investigations (5) and in contrast to others (10). *Kaplan & Tan* (10) thus found ANA in 14 of 21 patients with infectious mononucleosis. However, this may be attributed to the fact that they used undiluted serum for the demonstration of ANA or to the cryoprecipitability of ANA (10). It has been suggested (10) that the infective agent in infectious mononucleosis, i.e. probably the E-B virus (6), may provide the stimulus for a transient ANA formation. The formation of SMA in infectious mononucleosis might be analogous to this, but how it happens needs further investigation.

In a previous study of SMA of the IgG class (3). it was found that the staining of both gastric smooth muscle and renal glomeruli was abolished after absorption with crude actomyosin, suggesting that the same antibody reacted with both antigens. It was also found that antibodies yielded higher titres when tested on gastric smooth muscle than when tested on renal glomeruli. This may explain why glomerular antibodies could not be demonstrated in sera with SMA in low titres in this stud But according to this hypothesis, sera wi SMA in higher titres should be expected to react with both gastric smooth muscle and renal glomeruli. However, only a few sera with SMA of the IgG and the IgA class did react with renal glomeruli. This supports the view that antibodies with different specificities may occur (9, 12).

As regards antibodies of the IgM class, a high proportion of sera which reacted with stomach smooth muscle also reacted with renal glomeruli, and a correlation between the glomerular antibody titre and the SMA titre was revealed. Absorption of some sera with crude actomyosin prevented the staining of both stomach smooth muscle and renal glomeruli. This could indicate that, in some cases, the same antibody reacts with both the gastric smooth muscle and the renal glomeruli, and thus that IgM-SMA have a broader specificity than IgG and IgA smooth-muscle antibodies.

The author wishes to thank Dr. *E. Kjems*, Statens Seruminstitut, Copenhagen for valuable discussions and for providing the sera and performing the Paul-Bunnell reactions.

This investigation was supported by the *Danish Medical Research Council* (Grant No. 512-3064).

REFERENCES

1. *Ajdukiewicz, A. B., Dudley, F. J., Fox, R. A., Doniach, D. & Sherlock, S.:* Immunological studies in an epidemic of infective short-incubation hepatitis. Lancet *I:* 803–805, 1972.
2. *Andersen, P. & Beutner, E. H.:* Defined immunofluorescent studies of cytoplasmic antibodies. Int. Arch. Allergy *43:* 780–790, 1972.
3. *Andersen, P.:* Indirect immunofluorescence studies of smooth muscle antibodies. Acta path. microbiol. scand. Sect. B, *82:* 577–584, 1974.
4. *Beutner, E. H., Sepulveda, M. R. & Barnett, E. V.:* Quantitative studies of immunofluorescent staining. Bull. Wld Hlth Org. *39:* 587–606, 1968.
5. *Carter, R. L.:* Antibody formation in infectious mononucleosis. Brit. J. Haemat. *12:* 268–275, 1966.
6. *Henle, G., Henle, W. & Diehl, V.:* Relation of Burkitt's tumor-associated herpes-type virus to infectious mononucleosis. Proc. Natl. Acad. Sci. U.S.A. *59:* 94–101, 1968.
7. *Holborow, E. J., Hemsted, E. H. & Mead, S. V.:* Smooth muscle autoantibodies in in-

fectious mononucleosis. Brit. med. J. *3:* 323–325, 1973.

8. *Johnson, G. D., Holborow, E. J. & Glynn, L. E.:* Antibody to smooth muscle in patients with liver disease. Lancet *II:* 878–879, 1965.
9. *Johnson, G. D., Holborow, E. J. & Glynn, L. E.:* Antibody to liver in lupoid hepatitis. Lancet *II:* 416–418, 1966.
10. *Kaplan, M. E. & Tan, E. M.:* Antinuclear antibodies in infectious mononucleosis. Lancet *I:* 561–563, 1968.
11. *Kristensen, M.:* Studies of the sero-diagnosis of mononucleosis infectiosa. Acta path. microbiol. scand. Suppl. *37:* 339–356, 1938.
12. *Trenchev, P., Sneyd, P. & Holborow, E. J.:* Immunofluorescent tracing of smooth muscle contractile protein antigens in tissues other than smooth muscle. Clin. exp. Immunol. *16:* 125–136, 1974.
13. *Whitehouse, J. M. A. & Holborow, E. J.:* Smooth muscle antibodies in malignant disease. Brit. med. J. *4:* 511–513, 1971.
14. *Whittingham, S., Mackay, I. R &Irwin, J.:* Autoimmune hepatitis Immunofluorescence reactions with cytoplasm of smooth muscle and renal glomerular cells. Lancet *II:* 1333–1335, 1966.
15. *Wright, R.:* Australia antigen and smooth muscle antibody in acute and chronic hepatitis. Lancet *I:* 521–522, 1970.

Acta path. microbiol. scand. Sect. C, *83:* 28–34, 1975

AGGLUTINABILITY OF SOME SELECTED STREPTOCOCCI BY IMMUNE COMPLEXES

POUL CHRISTENSEN

The Department of Medical Microbiology, University of Lund, Sölvegatan 23, S-223 62 Lund, Sweden

Christensen, P. Agglutinability of some selected streptococci by immune complexes. Acta path. microbiol. scand. Sect. C, *83:* 28–34, 1975.

It is shown that some selected streptococci are agglutinable by soluble antigen-rabbit antibody complexes owing to the interaction between the streptococci and immunoglobulins, irrespective of the antibody combining sites. Increased agglutination titer of rabbit anti-egg albumin serum for the streptococci was obtained by addition of egg albumin, but not bovine or human albumin. The agglutination of the streptococci was diminished by addition of preimmune serum or by *trypsin treatment of the streptococci; trypsin treatment diminishes the capacity of the streptococci to take up* ^{125}*I labelled IgG myeloma protein. The effect of variation of the egg albumin concentration and the effect of the ratio between antibody N and egg albumin N was shown.* The possible mechanism of the agglutination of the streptococci by immune complexes is suggested.

Key words: Streptococci; agglutinability; immune complexes.

P. Christensen, Department of Medical Microbiology, Sölvegatan 23, S-223 62 Lund, Sweden.

ceived 20.vii.74 Accepted 27.vii.74

The interaction between some streptococci d immunoglobulins, irrespective of the antibody combining sites, can be demonstrated by the ability of these streptococci to agglutinate sheep red cells, coated with a subagglutinating dose of rabbit anti-sheep red cell antibodies (*Kronvall* 1973, *Christensen* & *Kronvall* 1974).

This paper concerns the agglutinability of some selected streptococci by soluble antigen-rabbit antibody complexes.

MATERIALS AND METHODS

Streptococcal Strains

Three streptococcal strains capable of taking up a relatively large amount of ^{125}I labelled IgG myeloma proteins (*Christensen* & *Oxelius* 1974), but not agglutinated by IgG myeloma proteins (0.5 mg/ml) were used. The purpose of the latter requirement was to avoid strains agglutinated by IgG by the interaction between IgG and streptococci, irrespective of the antibody combining sites; *some streptococci are agglutinated* by purified IgG myeloma proteins (*Christensen*, unpublished observation).

Streptococcus group A, type M 1 (No 8198) was kindly supplied by the Central Publis Health Laboratories, Colindale, London. The group C strain and the group G strain used were obtained from routine bacteriological specimens; these strains were group in the way described previously (*Christensen et al.* 1973).

The streptococci were cultured on Todd Hewitt Broth (Sigma). A stock suspension of 2.5×10^{10} streptococci/ml PBS (*phosphate buffered saline, 0.12 M NaCl, 0.03 M phosphate, pH 7.2*) was prepared as described previously (*Christensen* & *Oxelius* 1974).

Trypsin Treatment of the Streptococci

Trypsin treatment was performed to homogenize the streptococci for the agglutination experiments

(*Allison;* described by *Rudd et al.* 1939). Trypsin treatment reduces the ability of the streptococci to take up ^{125}I labelled IgG myeloma protein (*Christensen* & *Oxelius* 1974); unless otherwise stated, the minimal amount of trypsin, necessary to homogenize the streptococci was used This was about 1 mg trypsin (type III, Sigma, St. Louis, M. O. U.S.A.) per 10 ml of streptococcal stock suspensions of the three strains used in the experiments. The streptococci were suspended in PBS and incubated with trypsin at 37° C for 1 h. After incubation of the suspensions, 5 mg soya bean trypsin inhibitor (Sigma, type I-S) was added, after which the mixture was washed three times in PBS.

Estimation of the Ability of the Trypsin-Treated Stock Suspensions of Streptococci to Take up IgG, Irrespective of the Antibody Combining Sites

The IgG myeloma protein was purified, labelled with ^{125}I and the ability of 0.2 ml of the trypsin-treated streptococcal stock suspensions to take up ^{125}I labelled IgG myeloma protein was determined as described previously (*Christensen* & *Oxelius* 1974).

Albumins and Antisera

Egg albumin, bovine serum albumin and human serum albumin were purchased from Sigma. Solutions were prepared in PBS. Antisera against the albumins were raised in rabbits; 0.5 mg albumin, suspended in Freund's adjuvans, was injected subcutaneously and twice at an interval of 3 weeks.

Sera obtained from the same rabbits before immunization were used in the inhibition experiments.

The content of egg albumin antibody in the rabbit anti-egg albumin serum, as measured with the method described by *Heidelberger* & *Kendall* (1929, 1935a, b and c and 1937) was 640 µg N/ml serum; the protein was measured with a modification of *Folin's* method (*Lowry et al.* 1951).

Agglutination Method

The albumins or the antisera were diluted 1:2, 1:4, 1:8 etc in PBS in Microtiter plates (V-shape) (Cooke, Zollikon, Switzerland). 25 µl of the stock suspensions of the trypsin-treated streptococci was deposited in each well and the sedimentation pattern was read after 18 h at 20° C. A negative control with streptococci and PBS was included in every test. Agglutination was recorded as such when a layer of streptococci covered the bottom of the well.

Soluble complexes between antigen and antibodies form in the presence of antigen excess (*Heidelberger* & *Kendall* 1929, 1935 a, b and c and 1937). The following experiments were performed to ascess the agglutinability of the selected streptococci by soluble immune complexes:

Rabbit anti-egg albumin serum (25 µl) was diluted 1:2, 1:4, 1:8 etc in PBS, the first well containing 0.80 µg antibody N. In each well was deposited 25 µl egg albumin solution, containing 0.06 µg N. The ratio of the antibody N to egg albumin N in the first well was then 13.3:1 (antibody excess; equivalence at 9.7:1 (*Heidelberger* & *Kendall* 1935)), in the second 6.7:1 (antigen excess), etc. 25 µl trypsin-treated streptococcal stock suspension was finally added to each well.

RESULTS

Agglutinability of Some Selected Streptococci by Soluble Immune Complexes

Streptococcus group A, type M 1, group C, strain 81 C and group G, strain 113 G were agglutinated by dilutions of rabbit anti-egg albumin serum, to which a constant amount of egg albumin (0.06 µg N) was added in antigen excess, as are given in Fig. 1 and Table 1.

The agglutination titer rose substantially after addition of egg albumin; the strains were not agglutinated by the egg albumin solution (2 µg N/ml-0.01 µg N/ml tested).

Effect of the Ratio of Antibody N to Egg Albumin N and the Dilution of Rabbit Anti-Egg Albumin Serum on the Agglutination of Streptococcus Group C, Strain 81 C by Soluble Immune Complexes

In the experiments described above, group C, strain 81 C was agglutinated by antibody-egg albumin complexes corresponding to a dilution of rabbit anti-egg albumin serum of 1:256; in order to ascertain whether the failure to agglutinate in higher titers was caused by too low a concentration of antibodies *or* complexes being too small in the presence of the antigen in extreme excess (ratio of the antibody N: egg albumin N 1:9.6) the following experiments were performed.

Rabbit anti- egg albumin serum and egg albumin were diluted by a "chess-board" ti-

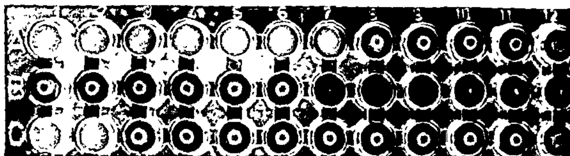


Fig. 1. The Agglutination of Streptococcus Group C, Strain 81 C by Soluble Immune Complexes. 25 µl rabbit anti- egg albumin serum was diluted 1:2, 1:4, 1:8 etc, 25 µl egg albumin (0.06 µg N) was then added to each well, followed by 25 µl streptococcal stock suspension. Agglutination occurred at a 1:256 dilution of serum (well marked "8", row "A"). 25 µl egg albumin (1 µg N) diluted 1:2, 1:4, 1:8 etc did not agglutinate the streptococci (row "B"), while anti- egg albumin serum agglutinated at a dilution of 1:4 (row "C", well marked "2").

TABLE 1. *The Agglutination of Some Selected Streptococci by Soluble Immune Complexes*

Bacterial strain	Solution, added to each dilution of antiserum*	Highest dilution of rabbit anti-egg albumin serum, producing agglutination	Ratio antibody N: egg albumin N in the highest dilution of antiserum, producing agglutination
Group A, type M 1	0.06 µg egg albumin N (25 µl)	1:64	1:2.4
	25 µl PBS	1:4	–
Group C, strain 81 C	0.06 µg egg albumin N (25 µl)	1:256	1:9.6
	25 µl PBS	1:4	–
Group G, strain 113 G	0.06 µg egg albumin N (25 µl)	1.128	1·4.8
	25 µl PBS	1:4	–

* None of the strains were agglutinated by the egg albumin solution

tration; the content of antigen and antiserum in the single well is shown in Fig. 2.

Independently of the antibody N: egg albumin N ratio, agglutination occurred at a 1:256 dilution of rabbit anti-egg albumin serum (Table 2).

In another experiment, the amount of antigen in the wells in row "A", Fig. 2, was reduced to 0.01 µg N. The first four wells then contained antibodies against egg albumin in excess. The results of the "chess-board"—titration is given in Table 3. Agglutination was seen in the dilution rows from the fourth and fifth well (equivalence between these two wells) in row "A", corresponding to a 1:256 dilution of rabbit anti-egg albumin serum (egg albumin 0.001 µg N in this well: row "E", well no. 4).

Effect of Serum, Obtained before Immunization with Egg Albumin, on the Agglutinability of Streptococcus Group C, Strain 81 C, by Soluble Egg Albumin-Antibody Complexes

Dilution of egg albumin and rabbit anti-egg albumin serum was done as shown in Fig. 2. 25 µl rabbit serum, diluted 1:10 and ob-

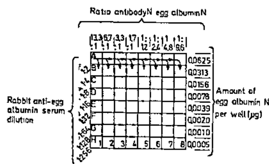


Fig. 2. "Chess-board"- Titration of Egg Albumin and Rabbit Anti- Egg Albumin Serum.
25 µl serum was diluted 1:0, 1:2, 1:4, 1:8 etc in PBS (left- to- right, row "A"). To each well was then added 25 µl egg albumin solution, containing 0.1250 µg N). Each well in row "A" was then diluted 1:2, 1:4, 1:8 etc. Finally, 25 µl trypsin-treated streptococcal stock suspension was added to each well, and the agglutination was read after 18 h at room temperature.

TABLE 2. *Effect of the Antibody N: Egg Albumin N Ratio on the Agglutination of Streptococcus Group C, Strain 81 C by Immune Complexes*

	1	2	3	4	5	6	7	8
A*	+	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+	–
C	+	+	+	+	+	+	–	–
D	+	+	+	+	+	–	–	–
E	+	+	+	+	–	–	–	–
F	+	+	+	–	–	–	–	–
G	+	+	–	–	–	–	–	–
H	+	–	–	–	–	–	–	–

For explanation, see Fig. 2.
+ = agglutination.
– = no agglutination.
* Antigen content in row "A": 0.06 µg N/well.

tained before immunization with egg albumin was deposited in each well, to which was afterwards added the streptococci. The highest dilution of rabbit anti-egg albumin serum capable of agglutinating the streptococci was then 1:16, compared with 1:256 in the experiment with PBS instead of rabbit preimmune serum.

TABLE 3. *Effect of the Antibody N: Egg Albumin N Ratio on the Agglutination of Streptococcus Group C, Strain 81 C by Immune Complexes*

	1	2	3	4	5	6	7	8
A*	+	+	+	+	+	–	–	–
B	+	+	+	+	+	–	–	–
C	–	–	–	+	+	–	–	–
D	–	–	–	+	+	–	–	–
E	–	–	–	+	–	–	–	–
F	–	–	–	–	–	–	–	–
G	–	–	–	–	–	–	–	–
H	–	–	–	–	–	–	–	–

For explanation, see Fig 2.
+ = agglutination.
– = no agglutination.
* Antigen content in row "A": 0.01 µg N/well.

Effect of Variation of the Antigen Concentration on the Agglutinability of the Selected Trypsin-Treated Streptococci by Immune Complexes

The minimal amount of egg albumin, necessary to increase the agglutination titer of rabbit anti-egg albumin serum from 1:4 to 1:8 for streptococcus group C, strain 81 C, was determined in the following experiment.

Egg albumin solution (25 µl), containing 3.12 µg albumin N, was diluted 1:2, 1:4, 1:8 etc. 25 µl rabbit anti-egg albumin serum, diluted 1:8 and 25 µl trypsin-treated stock suspension of group C, strain 81 C was then deposited in each well. The streptococci were agglutinated by addition of egg albumin from 1.56 to 0.006 µg N.

Effect of Trypsin Treatment on the Agglutinability of Group C, Strain 81 C Streptococci by Immune Complexes

The ability of group C, strain 81 C streptococci to take up ^{125}I-labelled IgG myeloma protein was reduced by further trypsin treatment of the stock suspension, homogenized with a minimal dose of trypsin. An increasing amount of trypsin was added to the stock suspension (Table 4), which was then incubated at 37° C for 1 h. The effect of the trypsin was stopped by addition of soyabean trypsin inhibitor. The ability of 0.2 ml stock

TABLE 4. *The Effect of Trypsin Treatment of Streptcoccus Group C, Strain 81 C, on the Agglutination, by Egg Albumin, of Streptococci Coated with Anti-Egg Albumin Antibodies*

Trypsin (mg) ded to 1 ml stock *suspension of* group C streptococci*	Uptaket of ^{125}I labelled IgG myeloma protein by *0.2 ml streptococcal stock* suspension (per cent) (of 1 μg added)	Agglutination by egg albumin of streptococci, coated with a subagglutination dose of rabbit-egg albumin antibodies** μg egg albumin N/well									
		1.56	0.78	0.39	0.18	0.09	0.05	0.02	0.01	0.006	0.0
None	38	(+)	+	+	+	+	+	+	(+)	(+)	–
0.3	29	–	(+)	+	+	+	+	+	+	–	–
0.6	28	–	(+)	+	+	+	+	+	+	–	–
1.2	25	–	–	+	+	+	+	+	+	–	–
10.0	12	–	–	+	+	+	+	+	+	–	–

* *Homogenized with a minimal dose of trypsin.*
** 1 ml trypsin-treated streptococcal stock suspension was left at room temperature with 1 ml rabbit anti-egg albumin serum, diluted 1:8 in PBS, then washed and suspended in 1 ml PBS.
+ = agglutination. No bacterial pellet.
(+) = agglutination. A layer of streptococci covering the bottom of the well and a *bacterial pellet* ("buttom").
– = *no agglutination.*

suspension to take up labelled IgG myeloma protein fell from 38 per cent to 12 per cent of 1 μg.

The amount of egg albumin, capable of increasing the agglutination titer of the rabbit anti-egg albumin serum from 1:4 to 1:8 was determined in the way described above. The stock suspension, 0.2 ml of which was capable of taking up 38 per cent of 1 μg IgG myeloma protein added, was agglutinated by anti-egg albumin serum diluted 1:8 and egg albumin 1.56–0.006 μg N per well; but when the uptake fell to 12 per cent, the range of the egg albumin decreased to 0.39–0.01 μg N.

Effect of the Specificity of the Antigen-Antibody Reaction on the Agglutination of Trypsin-Treated Streptococci by Immune Complexes

No increase in agglutination titer of rabbit anti-egg albumin serum for streptococcus group C, strain 81 C was seen after addition of bovine or human serum albumin (2 μg N/ml–0.01 μg N/ml). Addition of bovine serum albumin, 0.06 μg N to each well increased the agglutination titer of rabbit anti-bovine albumin serum from 1:2 to 1:64 for streptococcus group C. Addition of human serum albumin or egg albumin (2 μg N/ml–0.01 μg N/ml) caused no such increase. Similar results were obtained with rabbit anti-human albumin serum.

DISCUSSION

The agglutinability of the three selected streptococcal strains, streptococcus group A, type M 1, group C, strain 81 C and group G strain 113 G by immune complexes was demonstrated by an increased agglutination titer of rabbit anti-egg albumin serum after addition of egg albumin. Corresponding results were obtained for strain 81 C with

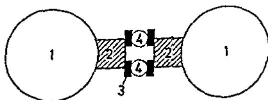


Fig. 3. Schema for the Agglutination of Streptococci by Complexes of Soluble Antigens and Antibodies.
1. Streptococci. 2. *The part of the immunoglobulin, not consisting of the antibody combining sites.* 3. The antibody combining sites. 4. Soluble antigen.

rabbit anti-bovine albumin serum/bovine albumin and rabbit anti-human albumin serum/human albumin.

The results lend support to the assumptions illustrated in the schema in Fig. 3. The increased agglutination titer of rabbit anti-egg albumin serum for strain 81 C required reaction between the antibody combining sites and their corresponding soluble antigens (between "3" and "4"); addition of other antigens produced no increase in the titer. The agglutination titer decreased also when the reaction of the streptococci with the anti-egg albumin antibodies, irrespective of the antibody combining sites (between "1" and "2") was partly inhibited by addition of preimmune serum or reduced by trypsin treatment of the streptococci.

Immune complexes in sera can be demonstrated by precipitation by Clq in gel diffusion (*Agnello et al.* 1969, 1970 and 1971), by the ultracentrifugal pattern of the serum (*Franklin et al.* 1957), chromatography on Sephadex G-200 (*Sulitzeanu et al.* 1964), histamine released from isolated perfused guinea pig lung (*Baumal* & *Broder* 1968), the "Farr-technique" (*Farr* 1958) and the platelet aggregation technique (*Penttinen et al. 1969)*. The use of these streptococci for detecting immune complexes in human sera would require: 1) elimination of antibodies, directed against the streptococcal test strain, from the sera under test and 2) an appropriate antigen-antibody ratio. However, adsorption of serum by any of the strains used in this investigation would remove some IgG from serum by means of the interaction between the streptococci and IgG, irrespective of the antibody combining sites (see *Christensen* & *Oxelius* 1974); it is not known whether condition 2) are met in immune complex diseases.

Streptococcal L-agglutination with group A, type M1 is used to detect anti-immunoglobulin antibodies in rheumatoid arthritis sera (*Kalbak* 1948, *Knöll* & *Tanner* 1968, *Otto et al.* 1971) and there is evidence that the agglutinating power resides in the IgG fraction of such sera (*Thulin* 1955, *Knöll* & *Tanner* 1968, *Otto et al.* 1971). Observations made in the present investigation suggest that type M 1 is agglutinated by complexes of immunoglobulins and anti-immunoglobulins of the IgG class. This possibility is receiving further attention.

REFERENCES

Agnello, V., Winchester, R. J. & Kunkel, H. G.: Detection of γ globulin complexes in patients with connective tissue disease by precipitation with Clq. Arthr. and Rheum. *12:* 654, 1969 (Abstract).

Agnello, V., Winchester, R. J. & Kunkel, H. G.: Precipitation reactions of the Clq component of complement with aggregated γ-globulin and immune complexes in gel diffusion. Immunol. *19:* 909–919, 1970.

Agnello, V., Koffler, D , Eisenberg, J. W., Winchester, R. J. & Kunkel, H. G.: Clq precipitins in the sera of patients with systemic lupus erythematosus and other hypocomplementic states: characterization of high and low molecular types. J. Exp. Med. *134:* 228s–241s, 1971.

Baumal, R. & Broder, I.: Studies into the occurrence of soluble antigen–antibody complexes in disease. I. A biological assay for soluble antigen-antibody complexes. Clin. Exp. Immunol. *3:* 525–536, 1968.

Christensen, P., Kahlmeter, G., Jonsson, S. & Kronvall, G.: New method for the serological grouping of streptococci with specific antibodies adsorbed to protein A-containing staphylococci. Inf. Immun. *7:* 881–885, 1973.

Christensen, P. & Kronvall, G.: Capacity of group A, B, C, D and G streptococci to agglutinate sensitized sheep red cells. Acta path. microbiol. scand. Sect. B, *82:* 19–24, 1974.

Christensen, P. & Oxelius, V.-A.: Quantitation of the uptake of human IgG by some streptococci groups A, B, C and G. Acta pathol. microbiol. scand. Sect. B, *82:* 475–483, 1974.

Farr, R. S.: A quantitative immunological measure of the primary interaction between I* BSA and antibody. J. Infect. Dis. *103:* 239–262, 1958.

Franklin, E., Müller-Eberhard, H., Holman, H. & Kunkel, H. G.: An unusual protein component of high molecular weight in the serum of certain patients with rheumatoid arthritis. J. Exp. Med. *105:* 425–438, 1957.

Heidelberger, M. & Kendall, F. E.: A quantitative study of the precipitin reaction between type III pneumococcus polysaccharide and purified homologous antibody. J. Exp. Med. *50:* 809–823, 1929.

Heidelberger, M. & *Kendall, F. E.:* The precipitin reaction between type III *pneumococcus* polysaccharide and homologous antibody. I. A quantitative study and a theory of the reaction mechanism. J. Exp. Med. *61:* 563–591, 1935(a).

Heidelberger, M. & *Kendall, F. E.:* A quantitative theory of the precipitin reaction. II. A study of an azoprotein system. J. Exp. Med. *62:* 467–483, 1935(b).

Heidelberger, M. & *Kendall, F. E.:* A quantitative theory of the precipitin reaction. III. The reaction between crystalline egg albumin and its homologous rabbit antisera. J. Exp. Med. *65:* 697–720, 1935(c).

Heidelberger, M. & *Kendall, F. E.:* A quantitative theory of the precipitin reaction. IV. The reaction of pneumococcus specific polysaccharides with homologous rabbit antisera. J. Exp. Med. *65:* 647–660, 1937.

Kalbak, K.: Agglutination of haemolytic streptococci (group A) in serum from patients with rheumatoid arthritis. Acta med. scand. *130:* 358–370, 1948.

Knöll, P. & *Tanner, E.:* Serologische Studie über typenspezifische Antikörper gegen T-Proteine der hämolysierenden A-Streptokokken bei Polyarthritis Chronica Progressiva und degenerativen Gelenk- und Wirbelsäulerkrankungen. Z. Immun. Forsch. *136:* 22–31, 1968.

Kronvall, G.: A surface component in group A, C and G streptococci with non-immune reactivity for immunoglobulin G. J. Immunol. *111:* 1401–1406, 1973.

Lowry, O. H., *Rosebrough, N. J.*, *Farr, L. A.* & *Randall, R. J.:* Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193:* 265–275, 1951.

Otto, R., *Reske, W.*, *Venner, M.* & *Schröder, D.:* Der simultannachweis von Rheumafaktor und Streptokokken-antikörpern im Serum durch trypsinbehandelte Streptokokken. Z. Immun. Forsch. *142:* 230–247, 1971.

Penttinen, K., *Myllylä, G.*, *Mäkelä, O.* & *Vaheri, A.:* Soluble antigen-antibody complexes and platelet aggregation. Acta path. microbiol. scand. *77:* 309–317, 1969.

Rudd, G V., *White, C.* & *Ward, H. K.:* Studies on haemolytic streptococci from human sources. Aust. J. Exp. Biol. Sci. *17:* 25–34, 1939.

Sulitzeanu, D., *Yagi, Y.* & *Pressman, D.:* Liver localizing antibodies—the limited contribution of Ag-Ab complexes. J. Immunol. *92:* 724–729, 1964.

Thulin, K. E.: The reaction of streptococcal agglutination relative to the RAS factor in rheumatoid arthritis. Acta rheum. scand. *1:* 1-21 1955.

Acta path. microbiol. scand. Sect. C, 83: 35–42, 1975

USE OF THE MIXED HAEMADSORPTION TECHNIQUE TO DEMONSTRATE LECTINS ADSORBED TO MONOLAYER CULTURES

J. Jonsson, Anita Östborn, Astrid Fagraeus and V. Skoog

Department of Immunology, Karolinska Institutet, School of Medicine, and
Department of Immunology, National Bacteriological Laboratory, Stockholm, Sweden

Jonsson, J., Östborn, Anita, Fagraeus, Astrid & Skoog, V. Use of the mixed haemadsorption technique to demonstrate lectins adsorbed to monolayer cultures. Acta path. microbiol. scand. Sect. C, 83: 35–42, 1975.

The radial diffusion mixed haemadsorption test was found to be a convenient, specific, sensitive and reproducible procedure by which to demonstrate the presence of phytohaemagglutinin (PHA) and concanavallin A (Con A) on the surface of monolayer cultures. When 100–500 µg/ml of PHA or Con A was included in the culture medium for 24 hours, an easily demonstrable concentration of the lectin was attached to the culture and remained so for at least 60 days. The heterogenicity of the commercial PHA preparations used was reflected by the fact that only the homologous antiserum gave filled haemadsorption zones with Difco PHA. An anti Wellcome PHA gave ring- or target zones with both the homologous and the Difco PHA. Also the anti-Difco PHA serum gave ring- or target zones with the Wellcome PHA. The ring zone effect obtained with Wellcome PHA, therefore, depended on the properties of this antigen and most probably on the fact that the Wellcome preparation was more homogenous and therefore contained less antigenic specificities than the Difco preparation.

Key words: Lectins; mixed haemadsorption; monolayer cultures.

J. Jonsson, M.D., Dept. of Immunology, National Bacteriological Laboratory, S-105 21 Stockholm, Sweden.

Received 10.vii.74 Accepted 27.xii.74

In an investigation reported earlier (11), phytohaemagglutinin P was shown to stimulate the reappearance of organ specific and some non-organ specific antigens on cells from the surface of which they had virtually disappeared as an effect of prolonged cultivation. This phenomenon was demonstrated by the indirect immunofluorescence and, preferably, by the mixed haemadsorption technique. During this investigation the mixed haemadsorption technique also proved itself convenient for demonstrating the presence of lectins on the surface of monolayer cultures. The procedure is sensitive, reproducible and easy to perform. It therefore seems worthy of a brief description.

MATERIALS AND METHODS

Lectins

Phytohaemagglutinin (PHA). Bacto-Phytohaemagglutinin P (control 555583 Difco Laboratories, Detroit, Mich. USA) and Phytohaemagglutinin purified (control K 6117 and 6165 Wellcome Re-

* Institut for Medical Genetics, University of Uppsala, Sweden

search Laboratories, Beckenham Kent, England) were used. The preparations were kept dry, sealed at +4° C, and dissolved in tissue culture medium immediately before use.

Concanavallin A (Con A). A preparation was kindly supplied by Pharmacia AB Uppsala, Sweden.

Tissue Cultures

Monolayer cultures were prepared in bottles as described earlier (6). Milk dilution and Falcon plastic bottles were used for mixed haemadsorption tests. For immunofluorescence tests the cells were dispersed by trypsin from Roux flasks (5).

Thyroid cultures were prepared from surgical specimens as described before (7). Age of the culture and presence or absence of organ antigens was found not to affect its affinity for PHA or Con A.

HeLa cell cultures, cynomolgus' and green monkey kidney cultures prepared in the same way as the thyroid cultures were kindly supplied by prof. *Ake Espmark*, Department of Virology, National Bacteriological Laboratory.

Antisera

Anti-PHA. Two rabbit anti-PHA sera were prepared as described elsewhere (18). Their titres against PHA and their specificity characters are described under results.

Anti-Con A. A rabbit anti-Con A serum was kindly supplied by Dr. *Peter Biberfeld*, Stockholm. Its properties appear in the section on results.

Organ specific anti-thyroid sera. The human anti-thyroid ring zone (Th 549/69) and filled zone sera (Th 366^{V}/65) were those used in a previous study (11). Their titres in the MHT were both 1/6400.

Human non-organ specifically reactive filled zone serum was also the same as that used before (9) (U68^{IV}/66). Its titre against thyroid cells in the MHT was 1/1600.

Anti HeLa cell sera. A cynomolgus monkey' and a rabbit anti-HeLa cell serum were used in most experiments as a control of the indicator system. They were prepared by repeated injections of HeLa cells without adjuvant and had titres of 1/3200 and 1/12800, respectively, against HeLa cells in the MHT.

Rabbit anti-human thyroid microsome serum. This serum was kindly supplied by prof. *Ivan F. Roitt*, Middlesex Hospital, London. Its titre against thyroid cells in the MHT was 1/25600.

The Mixed Haemadsorption Test (MHT)

The modification used in the present study was a radial diffusion mixed haemadsorption disc test in which monolayer cultures are covered by an agar layer. The antisera are set to diffuse from filter paper discs placed on the free agar surface. When reaching the antigen, the diffusing antibodies attach to it, forming circular zones that can be visualized by the aid of erythrocytes coated with antiglobulin so that they attach to the zones. The test was performed on milk dilution bottles and Falcon plastic bottles. For further details of the technique the reader is referred to earlier reports (4, 6, 7, 8, 9, 10).

The Indirect Immunofluorescence Test

Indirect immunofluorescence tests with dispersed cells were performed largely as described by *Möller* (15) using 1 million of cells dispersed by trypsin and washed twice in Tyrode's solution containing 1 per cent of normal sheep serum. This medium was also used for further washings and as a diluent of sera. Each lot of cell suspension was incubated for 30 min with 0.2 ml of test serum and after one washing for a further 30 min with a sheep antirabbit fluorescein conjugate prepared according to standard rules and fulfilling the requirements proposed for a standard conjugate (2). After one further washing, the cells were suspended in Tyrode's solution containing 1 per cent normal sheep serum and mounted under a sealed coverslip on microscopic slides. The preparations were examined in the Zeiss immunofluorescence microscope using a HBO-200 lamp, excitation filter Bg3 and barrier filter 44.

Stimulation with Phytohaemagglutinin

Confluent monolayer cultures were incubated for 24 hours at 37° C with growth medium containg different concentrations of phyto-haemagglutinin as described in a previous report (11). The cultures were then washed once with growth medium and left at 37° C with this medium for at least 24 hours before used. Incubations with Con A were performed in the same way. Growth medium was in all cases Hank's solution containg 0.5 per cent lactalbumin hydrolysate and 10 per cent normal bovine serum.

RESULTS

The specificity of the reactions of the anti-PHA and anti-Con A was tested on thyroid cultures that had been pre-incubated with 500 µg of PHA and Con A, respectively, and left with medium for 24 hours before testing. Anti-HeLa cell serum and normal rabbit serum were included as positive and negative controls. The results from such an experiment appear from Table 1. The rabbit anti-

PHA (Difco) reacted with cultures incubated with PHA, but not with those incubated with Con A or with plain medium. Correspondingly, the anti-Con A serum reacted with Con A incubated cultures, but not with cultures incubated with PHA or with plain medium. The titres (1/1600–1/3200) were moderate, about 1/4–1/16 of those given by the strong anti-HeLa cell serum. All zones obtained were of the filled variety (see below).

TABLE 1. *Demonstration of Phytohaemagglutinin P (PHA) and Concanavallin A (Con A) on Monolayer Cultures of Human Thyroid Cells by the mixed Haemadsorption Technique (Reciprocal Serum Titres)*

Test serum	Unexposed cultures	*Cultures exposed to PHA 500 µg/ml	*Cultures exposed to Con A 500 µg/ml
Rabbit anti-PHA serum	12.5	3200	12.5
Rabbit anti-Con A serum	6.25	12.5	1600
Rabbit anti-HeLa cell serum	12800	51200	12800
Normal rabbit serum	<3.125	12.5	<3.125

* The cultures were incubated with either of the lectins for 24 hours, washed once and then incubated with plain medium for a further 24 hours.

TABLE 2. *Demonstration of Phytohaemagglutinin P (PHA) on Thyroid Cells by the Indirect Immunofluorescence Technique*

Test serum	Unexposed cultures	Cultures exposed to PHA 500 µ/ml
Rabbit anti-PHA serum dil.	<5	≥5
Normal rabbit serum	<5	<5

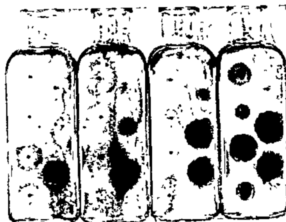
The cells were dispersed by trypsin from monolayer cultures pre-incubated with PHA as described for the MHT experiments in Table 1 and in parallel with these experiments.

In parallel with some of the experiments just described, PHA-stimulated and unstimulated cell cultures, respectively, were dispersed by trypsinization and tested against rabbit anti-PHA serum diluted 1/5 by the indirect immunofluorescence technique. In accordance with the mixed haemadsorption tests, PHA was traced specifically on the cells pre-incubated with PHA (Table 2).

The persistence of PHA P (Difco) on the cell surface was studied on thyroid cultures pre-incubated with PHA 500 µg/ml for 24 hours, washed and then left with medium at 37° C for varying lengths of time. Medium was usually changed once a week. Full reactivity was invariably obtained with anti-PHA serum still after 61 days (Table 3). However, if the pre-incubated human thyroid cells were dispersed by trypsinization and reseeded, no PHA could be demonstrated on the surface of the secondary monolayer cultures. The same result was invariably obtained after incubation with 100 and, less reproducibly, with 25 µg of PHA per ml.

PHA P (Difco) was attached to HeLa cells, cynomolgus kidney and green monkey kidney cultures in principally the same way as to human thyroid cultures. Human foetal lung cultures could not be tested because of their extreme sensitivity to the toxic effects of PHA. These cultures detached already after pre-incubation with 100 µg/ml of PHA P.

The connexion between the presence of PHA on the culture and the reappearance of cell surface antigen was studied in a number of experiments one of which is shown in Fig. 1. Two pairs of bottles with human thyroid cultures have been used, one pair (left) for testing the reappearance of surface antigen as demonstrated by organ specific human anti-thyroid sera and the other (right) for demonstrating the presence of PHA on the culture by the aid of rabbit anti-PHA sera. Within each pair, one (left) bottle is untreated and the other (right) pre-incubated with PHA P, 500 µg/ml. It appears that the faint zones produced by anti-thyroid and non-organ specific sera on the



Bottle 1 and 2

Ring zone serum	1/12.5	1/12.5	Normal human serum
	1/50	1/12.5	Non organ specifically reacting human serum
Filled zone serum	1/12.5	1/12.5	Cynomolgus anti HeLa cell serum
	1/50		

Bottle 3 and 4

Rabbit anti PHA (Wellcome) serum	1/12.5	1/12.5	Normal rabbit serum
	1/50	1/50	Rabbit anti HeLa cell serum
Rabbit anti PHA (Difco) serum	1/12.5	1/100	Rabbit anti human thyroid micro somal serum
	1/50		

Fig. 1. Reappearance of cell surface antigens on stimulation with PHA (bottle 1 and 2) and presence of PHA on the surface of stimulated cultures (bottle 3 and 4). Human thyroid cultures were used unstimulated and stimulated with PHA 500 µg/ml. The position of the tested sera appears from the diagram. For further details see text. (The faint reaction with normal human serum in bottle 2 is possibly due to an effluence from the adjacent ring zone serum disc. The cause for the reaction of normal rabbit serum with unstimulated culture in bottle 3 remains to be explained).

TABLE 3. *Duration of PHA Attachment on Human Thyroid Cultures Stimulated with 500 µg/ml*

Test serum	Reciprocal serum titre at different times after PHA stimulation — Number of days					
	1	12	27	35	54	61
Anti-PHA (Wellcome)	1600	1600	1600	200	1600	3200
Anti-PHA (Difco)	1600	6400	3200	3200	3200	6400
Anti-HeLa	25600	51200	51200	25600	51200	51200
Normal rabbit serum	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5

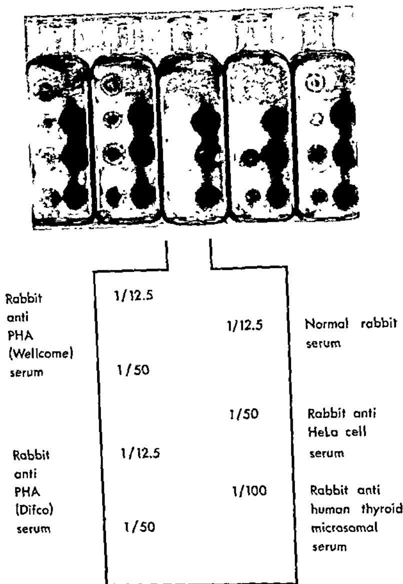


Fig. 2. Comparison of the reactivities of two different brands of PHA. Bottles 1 and 2 were treated with PHA Wellcome and bottles 4 and 5 with PHA Difco; bottles 1 and 4 with 100 µg/ml and 2 and 5 with 500 µg/ml. Bottle 3 is untreated. Position of sera is indicated in the diagram. For further explanations see text.

untreated culture are distinctly augmented on the PHA treated one as described before (11). The point of present interest is shown by the pair tested with rabbit anti-PHA sera and indicated for rabbit antibody. The presence of PHA on the pre-incubated right culture is clearly apparent from the zones produced by both anti-PHA sera (Wellcome at the top, Difco at the bottom (Fig. 1).

The two brands of PHA (Wellcome and P Difco respectively) used in the experiments were compared. The difference between them is demonstrated by Fig. 2. The figure shows two pairs of bottles with PHA-treated thyroid cultures with an untreated culture between them. The left pair was treated with PHA Wellcome and the right pair with PHA Difco 100 and 500 µg/ml, respectively.

TABLE 4. *Summary of Titres and Zone Types Produced in the Radial Diffusion Mixed Haemadsorption Test by Sera from Rabbits Immunized with PHA Wellcome and Difco, Respectively, when Tested Crosswise against these Two Antigens*

Anti-serum	*Reciprocal of titre and type of reactivity against*			
	PHA Wellcome		PHA Difco	
	100 µg/ml	500 µg/ml	100 µg/ml	500 µg/ml
Anti-PHA *Wellcome*	800 T	800 T	<12.5	400 T
Anti-PHA *Difco*	800 *T*	800 *T*	200 *F*	400 *F*

T = *target zone i.e. ring zone with a central core.*
F = *filled zone.*

The anti-PHA sera produced by immunizing with PHA Wellcome and Difco, respectively, were both found to give the "target" type of zones on cultures treated with PHA Wellcome. This type of zone was originally noted in reactions with thyroid cultures (7) and further analysed in a number of model systems (9, 10). The anti-PHA Wellcome serum reacted with target zones also on PHA Difco treated cultures while the anti-PHA Difco serum in this situation changed its zone pattern to the filled variety. Furthermore, both sera reacted on the culture treated with the lower dose (100 µg/ml) of PHA Wellcome while only the anti-PHA Difco serum reacted on the culture treated with the lower dose of PHA Difco. These findings are summarized in Table 4. Thus only the anti-PHA Difco serum reacting with its homologous antigen gave filled zones. Other combinations tested (Table 4) gave target zones.

DISCUSSION

It appears from the presented results that PHA and Con A can be demonstrated on monolayer cultures by their corresponding antisera in the same way as when a monolayer culture is pre-incubated with an antiserum against it. On the other hand, the lectins themselves, notably PHA, are comparable to an anti-culture serum since they are protein or glycoprotein in character reacting with structures on the culture cells (11, 12). The degree of resemblance of the lectin-surface-receptor union to an antigen-antibody link with regard to its immunochemical (3, 12, 13) character and its immunobiological (11, 14, 16, 17, 18) consequences is a matter of considerable theoretical interest. One feature of this union is notably apparent in the presented experiments, i.e. its extreme firmness of attachment which seems strong enough to outlive the culture.

The heterogenicity of the PHA preparations commercially available is shown by convincing evidence (1, 16, 19, 21) to which some of the results in this report may be added. They probably contain a mixture of reacting substances differeing in the affinity and specificity of the union and may therefore be regarded as a model for what happens when ordinary multispecific homologous antiserum is pre-incubated on the culture in the same way (9) or when a non-antibody protein like human serum albumin (HSA) or egg albumin (EA) is conjugated to the surface of a monolayer culture by the aid of bis diazotized benzidine (BDB) (10). Depending on the concentration and complexity of the attached material, the haemadsorption zones produced by antibodies to it in the radial diffusion mixed haemadsorption test will vary between a ring and a filled type. Attempts to analyse this phenomenon (9,

8. *Jonsson, J., Fagraeus, Astrid & Biberfeld, Gunnel:* The mixed haemadsorption technique as an aid to the diagnosis of auto-immune thyroid disease. Clin. Exp. Immunol. *3:* 287–304, 1968.
9. *Jonsson, J. & Fagraeus, Astrid:* On the mechanism of the ring zone effect obtained with the mixed haemadsorption technique. Studies with human anti-thyroid sera reacting with thyroid monolayer cultures. Immunology *17:* 387–411, 1969.
10. *Jonsson, J. & Fagraeus, Astrid:* On the mechanism of the ring zone effect obtained with the mixed haemadsorption technique. Studies with defined model systems. Acta path. microbiol. scand. Sect. B, *81:* 165–175, 1973.
11. *Jonsson, J., Fagraeus, Astrid & Sundqvist, K. G.:* Stimulation of organ specific and non-organ specific antigens on the surface of thyroid cells by phytohaemagglutinin. Scand. J. Immunol. *2:* 239–250, 1973.
12. *Kornfeld, S. & Kornfeld, R.:* Solubilization and partial characterization of a phyto-haemagglutinin receptor site from human erythrocytes. Proc. Nat. Acad. Sci. U.S.A. *63:* 1439–1446, 1969.
13. *Kornfeld, R. & Kornfeld, S.:* The structure of a phytohaemagglutinin receptor site from human erythrocytes. J. biol. Chem. *245:* 2536–2540, 1970.
14. *Lindahl-Kiessling, Kerstin & Mattsson, Anita:* Mechanisms of phytohaemagglutinin (PHA) action IV. Effect of some metabolic inhibitors on binding PHA to lymphocytes and the stimulatory potential of PHA pretreated cells. Exp. Cell Res. *65:* 307–312, 1971.
15. *Möller, G.:* Demonstration of mouse isoantigens at the cellular level by the fluorescent antibody technique. J. Exp. Med. *114:* 415–432, 1961.
16. *Naspitz, Ch. K. & Richter, M.:* The action of phytohaemagglutinin in vivo and in vitro, a review. Progr. Allergy *12:* 1–85, 1968.
17. *Punnet, T. & Punnet, H. H.:* Induction of leucocyte growth in cultures of human peripheral blood. Nature, Lond. *198:* 1173–1175, 1963.
18. *Skoog, V. T., Weber, T. H. & Richter, W.:* Studies on the interaction between mitogens and human lymphocytes in vitro. Exp. Cell Res. 1974. 85, 339–350, 1974.
19. *Spitz, M.:* Immunoelectrophoretic pattern of phytohaemagglutinin. Nature, Lond. *202:* 902–903, 1964.
20. *Sundqvist, K. G.:* Redistribution of surface antigens—a general property of animal cells? Nature New Biology *239:* 147–149, 1972.
21. *Weber, T., Nordman, C. T. & Gräsbeck, R.:* Separation of lymphocyte stimulating and agglutinating activities in phytohaemagglutinin from *Phaseolus vulgaris*. Scand. J. Haemat. *4:* 77–80, 1967.

Acta path. microbiol. scand. Sect. C, *83:* 43–51, 1975

IMMUNE RESPONSIVENESS TO *MYCOBACTERIUM LEPRAE* OF HEALTHY HUMANS

Comparison between Leucocyte Migration Inhibition, Lymphocyte Transformation, and Skin Testing

BJORN MYRVANG, KESETE NEGASSI, MONICA LOFGREN and TORE GODAL

Armauer Hansen Research Institute (AHRI)*, Addis Ababa, Ethiopia

Myrvang, B., Negassi, K., Lofgren, M. & Godal, T. Immune responsiveness to *Mycobacterium leprae* of healthy humans. Comparison between leucocyte migration inhibition, lymphocyte transformation, and skin testing. Acta path. microbiol. scand. Sect. C, *83:* 43–51, 1975.

Immune responsiveness to *Mycobacterium leprae* was studied in various groups of healthy humans. Contacts of leprosy patients responded significanctly more than non-contacts by the methods of leucocyte migration inhibition, lymphocyte transformation and early and late lepromin testing. By classifying responses of strengths found in non-contacts as negative, 71.2 per cent of medical attendants, the main category of contacts, were responders by the leucocyte migration inhibition test, 44.2 per cent by the lymphocyte transformation assay and 50.0 per cent by the early lepromin reaction. On the other hand, no degree of the late lepromin reaction was found solely in *M. leprae*-exposed people. While the assays of leucocyte migration inhibition, lymphocyte transformation and early lepromin testing thus may be considered useful for detection of healthy individuals exposed to *M. leprae,* the late lepromin reaction appears unsuitable as a measure of exposure. Besides the association of negative responses by leucocyte migration inhibition, lymphocyte transformation and early lepromin tests, there was in the group of non-contacts a significant quantitative correlation between early and late lepromin reactions. In the group of medical attendants significant correlations were observed between the results of all tests employed.

Key words: *Mycobacterium leprae;* immune responsiveness; leucocyte migration inhibition; lymphocyte transformation; skin testing.

B. Myrvang, Inst. for Experimental Medical Research, University of Oslo, Ullevål Hospital, Oslo, Norway.

Received 22.xi.74 Accepted 7.viii.74

In leprosy patients immune responsiveness to *Mycobacterium leprae*, tested by leucocyte migration inhibition, lymphocyte transformation, and delayed skin reactivity (early and late lepromin reaction), has been found to be associated in the sense that their strengths gradually decrease from the tuberculoid to the lepromatous end of the leprosy spectrum (14).

In healthy human beings the picture appears to be more complicated. Considering

* Affiliated to the All-Africa Leprosy & Rehabilitation Training Centre, Addis Ababa and the University of Bergen, Norway.

first people without prior contact with leprosy patients, these people have been found to give a uniformly negative response to *M. leprae* by the *in vitro* methods of leucocyte migration inhibition and lymphocyte transformation (7, 9, 13). Their early lepromin reaction (read after 48–72 hours) varies from one study to the other (1, 17, 18), while a majority have regularly been found to be positive in the late lepromin reaction (read after 3–4 weeks) (1, 16, 17, 18).

A large proportion of people with occupational or household contact with leprosy patients respond to *M. leprae* by the *in vitro* tests mentioned (7, 9, 13). This has been interpreted as showing that leprosy is more infectious than indicated by the prevalence of the disease. *In vitro* testing of immune responsiveness to *M. leprae* may therefore have epidemiological implications in leprosy research. Although perhaps not convincingly demonstrated (15), most studies indicate that the degree of both early and late lepromin reactions tends to be greater in contacts than in non-contacts (2, 5, 6, 16, 17).

In the present investigation, immune responsiveness to *M. leprae* by the methods of leucocyte migration inhibition, lymphocyte transformation and early and late lepromin testing was studied in the same groups of contacts and non-contacts of leprosy patients. The purpose was firstly to compare the *in vitro* tests in order to establish which one at present may be most suitable for detection of healthy individuals exposed to *M. leprae*, and secondly to evaluate the relationship of the early and late lepromin reactions to exposure to leprosy and to the results of the *in vitro* assays.

MATERIALS AND METHODS

Test Subjects

A total number of ninety three adults participated in the study. They were classified into four groups according to contact with leprosy patients.

Seventeen of the participants had no known contact with leprosy patients, although seven had lived in a leprosy endemic area for more than one year. Fifty-five of those examined were medical attendants dealing with leprosy patients. They had all, for different lengths of time, been in close contact with many leprosy patients. The third group consisted of nine non-medical staff members of a leprosy hospital who had rarely experienced close contact with patients. Finally, the study included twelve household associates of leprosy patients. A majority of these had a close relationship with one patient only. A more detailed description of the various groups is found in a preceding paper (13).

The number of individuals examined by the various methods is evident from Tables 1, 2, 3 and 4. Blood for the *in vitro* tests was always drawn before the skin test was performed. None of the participants reported to have been lepromin-tested before.

Antigens

Leprosy bacilli were obtained from skin biopsies from lepromatous leprosy patients as previously described (8). The same batch of bacilli, which was stored in small portions at −20° C, was used for most of the *in vitro* experiments.

M. leprae for lepromin was prepared in the same way, followed by autoclaving and adjustment of the concentration to 160 mill. bacilli per ml saline (10) preserved with 0.5 per cent phenol The suspension, stored at +4° C, was mixed throughly before use. All skin tests were done within 6 months of the date of preparation of the lepromin.

Leucocyte Migration Inhibition

The test was performed with micro-capillaries. The technique and the results are described in a preceding paper (13). The results reported here were obtained with 7.5×10^7 *M. leprae*/ml tissue culture medium.

Lymphocyte Transformation

The method used has been described in detail by *Godal et al.* (8). The blastogenic response to *M. leprae* was assessed morphologically The examiner did not know from whom the cells originated. The method has, in our hands, been found to correlate closely with the incorporation of radioactive thymidine (8, 14). The findings were included in a previous summary of the results obtained by this method (9).

Lepromin Testing

0.1 ml lepromin (i.e. 1.6×10^7 *M. leprae*) was injected intradermally into the volar surface of the forearm. The early lepromin reaction was recorded as the widest diameter of induration at 48 or 72 hours, while the late reaction was read after 3 to 4 weeks when the presence of ulceration also was noted. Both reactions were converted into the five grades recommended by WHO

(21). Under these criteria, an early reaction with diameter of less than 6 mm is graded as −, one of 6 to 10 mm as +−, one of 11 to 15 mm as +, one of 16 to 20 mm as + + and one of more than 20 mm as + + +. Similarly, a late reaction with diameter of induration of less than 3 mm is called negative, one of 3 and 4 mm is graded as +−, one of 5 to 7 mm as +, one of 8 to 10 mm as + + and one of more than 10 mm as + + +. Irrespective of diameter of infiltration, late reactions that ulcerate are graded as + + +.

Statistical Analyses

Variance was expressed as standard deviation, and the Wilcoxon two sample test (3) was used to analyse the differences observed between the various groups of participants. The significance of the difference in the proportion of individuals classified as reactors was measured by the chi-square test, using graphs (prepared by Miss *M. V. Musset*, Statistical Service Section, National Institute for Medical Research, London) based on Mainland tables (12). The relationship between the immune response to *M. leprae* in different tests was measured by the Spearman rank correlation coefficient (3).

RESULTS

The inhibition of leucocyte migration and the blastoid response induced by *M. leprae* in *vitro* in the four groups of [illegible] are shown in Tables 1 and 2 [illegible].

In the migration assay, the medical atten-dants responded significantly more [illegible] than either the non-contacts [illegible] or the administrative staff group [illegible]. The latter group responded [illegible] strongly than the non-contacts [illegible]. While the strongest responding [illegible] had a migration index of [illegible] of people in the other three groups [illegible] considerably stronger inhibition. [illegible] dices <0.800 were regarded as positive re-sponses, there were significantly more re-sponders among medical attendants and household contacts than among [illegible] ($p<0.01$).

The blastoid response to *M. leprae* [illegible] 2) was significantly lower in the [illegible] group than in the group of medical [illegible] ($p<0.01$), household contacts [illegible] or administrative staff ($p<$[illegible]). [illegible] ference between medical attendants and ad-ministrative staff was also statistically [illegible] cant ($p<0.01$). If the results [illegible] as percentage of responders, [illegible]

TABLE 1. M. leprae-*induced Leucocyte Migration Inhibition in Four Groups of* [illegible] *Contact with Leprosy Patients*

	Number tested	Mean migration index ± SE	[illegible]*
Non-contacts	17	0.911 ± 0.018	[illegible]
Medical attendants	52	0.738 ± 0.015	[illegible]
Administrative staff	9	0.816 ± 0.023	[illegible]
Household contacts	12	0.834 ± 0.044	[illegible]

* Migration indices <0.800 were regarded as positive.

TABLE 2. *Lymphocyte Transformation by* M. leprae *in Four Groups of People* [illegible] *with Leprosy Patients*

	Number tested	Mean blastoid response (%) ± SE	Per[illegible]	[illegible]
Non-contacts	17	0.18 ± 0.07	[illegible]	[illegible]
Medical attendants	52	3.44 ± 0.84	[illegible]	[illegible]
Administrative staff	9	1.92 ± 0.81	[illegible]	[illegible]
Household contacts	12	2.41 ± 0.73	[illegible]	[illegible]

* Responses >0.5 per cent regarded as positive. ** Responses >2.0 per cent [illegible]

TABLE 3. *Early Lepromin Reaction in Four Groups of People with Different Contact with Leprosy Patients*

	Number tested	WHO grades					Mean infiltration ± SE (mm)
		–	+–	+	++	+++	
Non-contacts	16	10	6				3.75 ± 0.83
Medical attendants	48	17	7	18	6		8.98 ± 1.35
Administrative staff	7	4	2	1			5.00 ± 2.52
Household contacts	4	1	1	1		1	10.25 ± 4.59

TABLE 4. *Late Lepromin Reaction in Four Groups of People with Different Contact with Leprosy Patients*

	Number tested	WHO grades				
		–	+–	+	++	+++
Non-contacts	15		2	6	1	6
Medical attendants	47		1	8	7	31
Administrative staff	8			3	2	3
Household contacts	4				1	3

tained were highly dependent on the threshold chosen for positivity. The greatest differences between the non-contacts and the other groups were found at 0.5 per cent as threshold value. A threshold of 2 per cent minimum had to be chosen if all non-contacts were to be classified as non-responders. This resulted in a considerable decrease in the proportion of responders in the other groups. However, at both threshold values there was a statistically significant difference in the percentage of responders between non-contacts and medical attendants ($p<0.01$) and between non-contacts and household associates ($p<0.01$, threshold 0.5 per cent and $p<0.05$, threshold 2.0 per cent).

The results of the early and late lepromin reactions are shown in Tables 3 and 4, respectively. The early reaction was weak in all non-contacts and the diameter of infiltration did not exceed 10 mm in anyone tested, giving only negative (–) or doubtful reaction (+–). Half of the medical attendants and the household contacts, and one out of seven administrative staff had reactions classified as positive (+, ++ or +++). The reactions of the medical attendants were significantly stronger than those of the non-contacts ($p<0.01$), but otherwise there were no statistically significant differences between the various groups.

None of the late lepromin reactions recorded were negative according to the WHO grading, and only two non-contacts and one medical attendant had reactions classified as doubtful. Although thirteen of the fifteen non-contacts examined showed positive responses, the medical attendants reacted significantly more strongly ($p<0.02$) because of the higher proportion of strongly positive reactions in this group.

In the group of non-contacts the migration indices and the blastogenic responses correlated in the sense that they were both negative in all subjects when 0.800 and 2.0 per cent were used as thresholds for positivity in the tests of leucocyte migration and lymphocyte transformation, respectively (Fig. 1). A similar association was found between the *in vitro* assays and the early lepromin test. There was no tendency for non-contacts with doubtful early lepromin reactions to show stronger *in vitro* responses to *M. leprae* than those with completely negative reactions. There was also no tendency for strongly positive late reactions to be associated with mi-

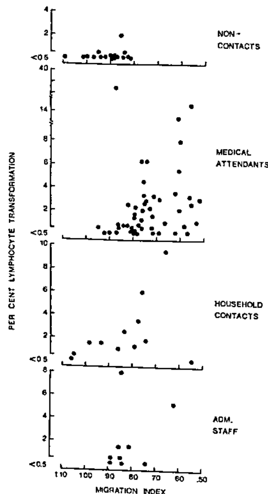


Fig 1. Comparison between responses to *M. leprae* by the methods of leucocyte migration inhibition and lymphocyte transformation in four groups of people with different degree of contact with leprosy patients.

gration indices and blastoid responses close to the thresholds chosen. Although all non-contacts, according to the WHO criteria, had negative or doubtful early lepromin reactions while they were almost uniformly positive in the late reaction, a significant correlation was found between the two recordings (R = 0.83, p<0.01), when analysis was done using diameter of infiltration for the early and WHO grades for the late reaction (Fig. 2).

A significant correlation (R = 0.42, p< 0.01) was found between migration and transformation values in the group of medical attendants (Fig. 1). If the results were simply grouped as positive or negative, the results obtained by the two methods correlated in 65 per cent of the medical staff. There was also a significant correlation between the early lepromin reaction and the *M. leprae*-induced migration inhibition (R = 0.35, p = 0.02) and blastoid response (R = 0.70, p<0.001). The histograms in Fig. 3 show the *in vitro* responses to *M. leprae* when the medical attendants were grouped according

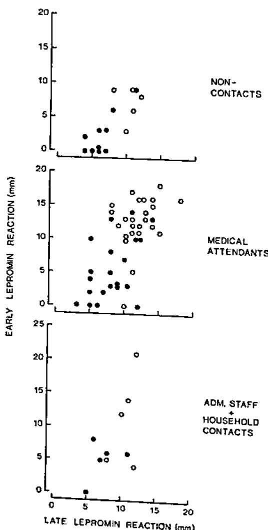


Fig. 2. Relationship between the early and late lepromin reaction in contacts and non-contacts of leprosy patients. Open dots refer to late reactions that ulcerated.

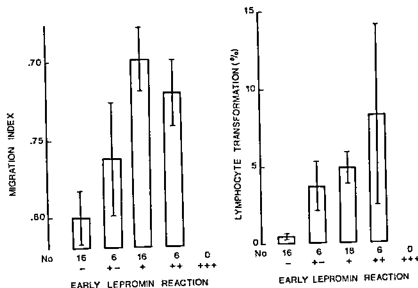


Fig. 3. Correlation between: Left: Early lepromin reaction (WHO grades) and *M. leprae*-induced inhibition of leucocyte migration, and Right: Early lepromin reaction and *M. leprae*-induced lymphocyte transformation in medical attendants dealing with leprosy patients. Bars show mean values, and lines on bars show standard error of the mean.

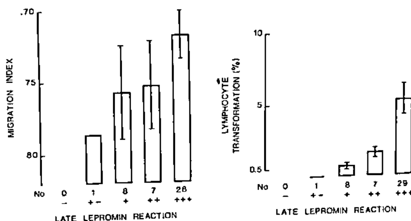


Fig 4. Correlation between: Left: Late lepromin reaction (WHO grades) and *M. leprae*-induced inhibition of leucocyte migration, and Right: Late lepromin reaction and *M. leprae*-induced lymphocyte transformation in medical attendants dealing with leprosy patients. Bars show mean values and lines on bars show standard error of the mean.

to the strength of their early lepromin reactions. In both *in vitro* assays, individuals with negative early lepromin reactions and the groups of negative and doubtful reactions combined showed significantly weaker responses than the positive skin reactors. If the results were simply considered positive or negative, results of the early lepromin skin test correlated with the tests of migration inhibition and lymphocyte transformation in 80 and 75 per cent of the cases, respectively. In this group of medical attendants (Fig. 4), the strength of the late lepromin reaction was also significantly correlated to both the inhibition of migration ($R = 0.34$, $p<0.05$) and to the blastoid response in particular ($R = 0.55$, $p<0.001$). Or considered in another way, occupational staff members with

strongly positive (+ + +) late lepromin reactions responded significantly more strongly to *M. leprae* in the transformation assay than the weak (+) reactors ($p<0.01$) and the weak (+) and intermediate (+ +) reactors taken together ($p<0.05$). Although positive late reactions were frequently combined with negative or doubtful early lepromin reactions, like in non-contacts, a statistically significant correlation ($R = 0.63$, $p<0.01$) was observed between the early and late recordings (Fig. 2).

In the small groups of administrative staff and household contacts there was no significant association either between results of the two in vitro assays (Fig. 1) or between the early and late lepromin reactions (Fig. 2). The blastoid response of the administrative staff group was, however, found to be significantly correlated to the early lepromin reaction ($R = 0.84$, $p<0.05$).

DISCUSSION

This study shows that people dealing with leprosy patients respond significantly more strongly to *M. leprae* than individuals not exposed to *M. leprae* both by the methods of leucocyte migration inhibition and lymphocyte transformation as well as in the early and late lepromin reaction. As argued in discussions of the application of the leucocyte migration inhibition test (13), this difference is likely to be due to sensitization of leprosy contacts with *M. leprae*. The main finding favouring this conclusion is that inhibition of leucocyte migration induced by BCG is similar in the contact and non-contact groups (13). Since the present study deals with the same groups of people, it seems reasonable that variations in exposure to *M. leprae*, by all test used is the factor mainly responsible for the differences observed between the groups. Therefore, since migration indices <0.800, blastogenic responses >2.0 per cent and early lepromin reactions >10 mm were never found in non-contacts, but frequently in contacts, we conclude that responses above these thresholds reflect sensitization with *M. leprae* itself. Accordingly, both the leucocyte migration inhibition test, the method of lymphocyte transformation and the early lepromin reaction may have implications as measures of exposure in leprosy.

In contrast, no strength of the late lepromin reaction was found solely in people who had experienced contact with leprosy patients. The most likely explanation of this discrepancy is that the late lepromin reaction not only is dependent on the initial level of delayed type immune response to *M. leprae*, as illustrated by correlation to early lepromin reactions and responses to *M. leprae in vitro* assays. In addition the test allows for an augmentation mechanism to take place before the test is read – 3 to 4 weeks after injection. Strong, late lepromin reactions are therefore also observed in people not exposed to *M. leprae*, although more rarely than in people with pre-existing delayed hypersensitivity due to natural sensitization. This makes the test unsuitable as a measure of exposure.

The finding of only negative and doubtful early lepromin reactions in people without contact with leprosy patients and positivity in a considerable proportion of contacts is compatible with *Fernandez'* original observations on this test (4, 5). Others have however found reactors also among people living in leprosy free areas (17, 18). This variability is not readily explained by differences in exposure to other mycobacteria (13), and we tend to think that the disparities may be attributed to difficulties in standardization in preparation, quantitation and reading of the test (10). The number of acid-fast bacilli is at present the only available method for quantitative standardization. Since it is most likely that the reaction is more dependent on the concentration of soluble antigens than on the number of acid-fast bacilli (2, 4, 16), another approach to standardization could give more consistent results.

The early lepromin results indicate that the sensitivity and specificity of the test may be inferior to the *in vitro* assays. Therefore, it is debatable whether or not the early lepromin test, with the crude lepromin used,

should be considered suitable for enumeration of *M. leprae*-exposed individuals. Since a skin test, however, in epidemiological studies would have practical advantages to the *in vitro* methods established, investigations on the specificity of modified lepromins that favour the early reaction (2, 4, 11) could be worthwhile. Moreover, the extensive growth of *M. leprae* in the armadillo (20) may now also make it feasible to explore the possibility of isolating *M. leprae*-specific fractions for skin testing.

As discussed in previous papers (7, 9, 13), both *in vitro* systems appear to have sufficient specificity to be useful as monitors for immune responses elicited by *M. leprae*. However, the specificity of the tests need careful attention in each case. Although in the present study most responders were revealed by the leucocyte migration inhibition test, it is not possible from the figures obtained to conclude which method is most suitable for detection of healthy individuals exposed to *M. leprae*. The short culture period and the non-requirement for sterility may favour the migration test, which however, is very antigen-consuming and also handicapped by not being fully understood in immunological terms (19).

The relationship of positivity in the various tests to resistance to leprosy remains largely unknown. Observations by *Dharmendra* & *Chatterjee*, cited by *Rees* (16), can lead one to believe that the late lepromin reaction might possibly recognize susceptibility in non-leprous persons. It has also been suggested that people showing immunological evidence of exposure by the lymphocyte transformation test are to be considered as healthy immune individuals (7). In spite of the significant correlations between blastoid responses, migration inhibitions and early lepromin reactions, the considerable disagreements do to some extent impede the interpretation of responders as resistant individuals. As in other systems, however, it is difficult to evaluate whether the disparities reflect immunological dissociations or can be accounted for by technical factors (19). The latter may create more problems when working with *M. leprae* than when working with other antigens, since the bacilli, frequently in clumps, are always contaminated with tissue material. Moreover, the use of autoclaved and phenol-preserved antigen *in vivo*, but not *in vitro*, may also have contributed to the observed dissociations.

The most likely interpretation of the high exposure figures is that subclinical infection appears to be the most common outcome of infection with *M. leprae* (7, 9, 13). A practical shortcoming of the methods employed is therefore that none of them detect individuals in the preclinical phase of the disease. To obtain a method of doing this, it might be worthwhile pursuing other approaches such as sensitive methods for detection of humoral immune responses to *M. leprae*.

We are grateful to the volunteers who made the study possible.

REFERENCES

1. *Bechelli, L. M., Quagliato, R., Nakamura, S. & Lima Filho, E. C.:* Lepromin test in non-contacts (mainly 1–19 years old) living in an area of low leprosy endemicity Int. J. Leprosy *39:* 136–145, 1971.
2. *Dharmendra:* The lepromin test. Belra Medical Series No. 1, London 1948
3. *Documenta Geigy:* Scientific tables 6. ed. J. R. Geigy S. A., Basle, Switzerland, 1962.
4. *Fernandez, J. M. M.:* The early reaction induced by lepromin. Int. J. Leprosy *8:* 1–14, 1940.
5. *Fernandez, J. M. M. & Olmos Castro, N.:* The early lepromin reaction. Study of antigens obtained from *Mycobacterium leprae*. Trop. Dis. Bull. *40:* 245–248, 1943
6. *Gehr, E. & Munder, H. M.* Die Lepromin-Reaction bei verschienenen Volksgruppen in Suriname. Z. Tropenmed. u. Parasit. *5:* 379–387, 1954.
7. *Godal, T., Løfgren, M. & Negassi, K:* Immune response to *M. leprae* of healthy leprosy contacts. Int. J. Leprosy *40*· 243–250, 1972.
8. *Godal, T., Myklestad, B., Samuel, D. R. & Myrvang, B.:* Characterization of the cellular immune defect in lepromatous leprosy: A specific lack of circulating *Mycobacterium leprae*-reactive lymphocytes. Clin. exp. Immunol. *9:* 821–831, 1971.

9. *Godal, T. & Negassi, K.:* Subclinical infection in leprosy. Brit. Med. J. II: 557–559, 1973.
10. *Hanks, J. H., Abe, M., Nakayama, T., Tuman, M., Bechelli, L. M. & Dominguez, V. M.:* Studies towards standardization of lepromin. Bull. Wld Hlth Org. 42: 703–709, 1970.
11. *Kitano, H.:* The Mitsuda reaction by vaccines treated with the ultrasupersonic wave. Int. J. Leprosy *9:* 29–38, 1940.
12. *Mainland, D., Herrara, L. & Sutcliffe, M. L.:* Statistical tables for use with binominal samples. New York University College of Medicine, 1956.
13. *Myrvang, B.:* Immune responsiveness to *Mycobacterium leprae* of healthy humans. Application of the leucocyte migration inhibition test. Acta path. microbiol. scand. Sect. B, *82:* 707–714, 1974.
14. *Myrvang, B., Godal, T., Ridley, D. S., Frøland, S. S. & Song, Y. K.:* Immune responsiveness to *Mycobacterium leprae* and other mycobacterial antigens throughout the clinical and histopathological spectrum of leprosy. Clin. exp. Immunol. *14:* 541–553, 1973.
15. *Newell, K. W.:* An epidemiologist's view of leprosy. Bull. Wld Hlth Org. *34:* 827–857, 1966.
16. *Rees, R. J. W.:* The significance of the lepromin reaction in man. Progr. Allergy *8:* 224–258, 1964.
17. *Rotberg, A., Bechelli, L. M. & Keil, H.:* The Mitsuda reaction in a nonleprous area. Int. J. Leprosy *18:* 209–220, 1950.
18. *Shepard, C. C. & Saitz, E. W.:* Lepromin and tuberculin reactivity in adults not exposed to leprosy. J. Immunol. *99:* 637–642, 1967.
19. *Special feature: In vitro* methods of cell-mediated immunity: A progress report. Cell. Immunol. *6:* 331–347, 1973.
20. *Storrs, E. E., Walsh, G. P., Burchfield, H. P. & Binford, C. H.:* Leprosy in the armadillo: New model for biochemical research. Science *183:* 851–853, 1974.
21. *World Health Organization:* WHO expert committee on leprosy. Wld Hlth Org. techn. Rep. Ser. no. 71, 1953.

Acta path. microbiol. scand. Sect. C, *83:* 52–58, 1975

DEMONSTRATION OF DELAYED-TYPE HYPERSENSITIVITY IN GUINEA-PIGS BY THE AGAROSE PLATE TECHNIQUE

O. Brændstrup, M. Magnusson, J. Maxild and O. Werdelin

The University Institute of Pathological Anatomy, University of Copenhagen, and the Tuberculin Department, Statens Seruminstitut, Copenhagen, Denmark

Brændstrup, O., Magnusson, M., Maxild, J. & Werdelin, O. Demonstration of delayed-type hypersensitivity in guinea-pigs by the agarose plate technique. Acta path. microbiol. scand. Sect. C, *83:* 52–58, 1975.

A recently developed method for demonstration of antigen-dependant migration inhibition, the agarose plate technique, and skin tests were used to demonstrate delayed hypersensitivity towards *Mycobacterium tuberculosis* and *Nocardia farcinica* in guinea-pigs. Peritoneal exudate cells from animals immunized with *M. tuberculosis* and *N. farcinica* displayed immunologically specific inhibition of migration after stimulation with tuberculin and *N. farcinica* sensitin, respectively. The slight cross reactivity between the two antigens demonstrated by skin tests was also observed with the agarose technique. Previous skin testing did not influence migration inhibition of peritoneal exudate cells. Migration inhibitory activity of supernatants of tuberculin stimulated lymph node cells from guinea-pigs which had been immunized with *M. tuberculosis* was demonstrated on peritoneal exudate cells from non-immunized animals.

Key words: Delayed-type hypersensitivity; agarose plate technique; guinea-pigs.

O Brændstrup, The University Institute of Pathological Anatomy, University of Copenhagen, Juliane Mariesvej 16, DK-2100 Copenhagen Ø, Denmark.

Received 9.x.74 Accepted 9.x.74

Inhibition of migration of cells from immunized animals in tissue culture by specific antigen was first demonstrated by *Rich & Lewis* (16). It is generally accepted that this phenomenon is an expression of cellular immunity, and the principle has been used in numerous studies. The assay most frequently applied is based on a method described by *George & Vaughan* (9) and modified by *David et al.* (6). The cells are enclosed in capillary tubes which are placed in vials containing a tissue culture medium. During culture the cells migrate from the capillary tubes on to the glass or plastic of the culture vial. The inhibition of the migration is caused by a soluble mediator, migration inhibitory factor (MIF), which can be demonstrated by allowing cells from non-immune individuals to migrate in culture supernatants of cells from immune individuals in which the cells have been stimulated with the relevant antigen (1, 7).

An agarose plate technique for *in vitro* demonstration of delayed-type hypersensitivity in guinea-pigs was first described by *Carpenter et al.* (2). The authors showed that a semisolid medium containing agar and guinea-pig serum in a tissue culture medium supported the migration of guinea-pig cells,

including fragments of lymph node and suspensions of peritoneal exudate cells (PEC). The cell suspensions were placed in holes cut in the agar gel. The migration took place predominantly between the agar layer and the petri dish containing the agar. Migration of cells from immunized guinea-pigs was specifically inhibited when antigen was added to the medium *Clausen* modified this technique for studies of antigen-induced migration inhibition of human peripheral blood leucocytes (3, 4), and for demonstration of MIF in supernatants from cultures of these cells (4).

In the present study, the migration of guinea-pig PEC was studied employing the agarose plate technique in *Clausen's* modification. Since purified protein derivative (PPD) of tuberculin prepared from cultures of *Mycobacterium tuberculosis* and sensitin prepared from cultures of *Nocardia farcinica* show only weak cross reactivity in delayed-type skin tests on immunized guinea-pigs (14), those preparations were used. The study was undertaken in order to determine whether the agarose plate technique is as specific as the skin test technique, and whether MIF in supernatants from immunized guinea-pig lymph node cells could be demonstrated by the agarose plate technique.

MATERIALS AND METHODS

Guinea-pigs. The animals used were random-bred, male, albino guinea-pigs, strain Ssc:AL from Statens Seruminstitut, weighing 400 g to 500 g.

Immunization: 37 guinea-pigs were immunized intracutaneously (i.c.) in four sites on the abdomen with 0.1 ml of a suspension of dried, heat-killed cells of *M. tuberculosis* strain 3 in light paraffin oil (Marcol 52). 25 animals were immunized in the same way with a suspension in the same vehicle of dried, heat-killed cells of *N. farcinica* strain 84 (15). Both suspensions contained 40 µg of organisms per 0.1 ml. 23 non-immunized guinea-pigs served as controls.

Skin tests: Three weeks later 18 animals sensitized with *M. tuberculosis*, 18 animals sensitized with *N. farcinica* and 12 non-immunized guinea-pigs were given double i.c. injections of 0.1 ml of dilutions of purified tuberculin RT 23 (13) and *N. farcinica* sensitin RS 84 (15) containing 20 µg and 2 µg per ml, respectively (12). The eight injections were distributed at random in each animal and readings were performed after 24 hours, as reported previously (11).

Preparation of agarose plates: A sterile 1.8 per cent solution of agarose (Litex, Glostrup, Denmark) in distilled water cooled to 47° C was mixed with tissue culture medium (TC-199, 10 x, Difco Laboratories, Detroit, Michigan, USA). Serum from non-immunized guinea-pigs (NGPS) and sterile water was added to a final concentration of 0.8 per cent agarose and 10 per cent NGPS in TC-199, single strength. Penicillin and streptomycin were added to achieve a final concentration of 10 IU per ml and 10 µg per ml, respectively. Sodium bicarbonate (Difco Laboratories) was added so that the pH of the medium after incubation at 37° C in 5 per cent CO_2 in air was 7.2–7.4. 5 ml of this medium was poured into sterile 50 mm plastic petri dishes (Millipore Filter Corp., Bedford, Mass. USA). After the medium had solidified, six holes with a diameter of 2.3 mm were cut in the gel and the plates were incubated at 37° C in 5 per cent CO_2 in air until use.

Antigens for stimulation of cells: Purified tuberculin RT 32 was prepared in 1970–71 in the same laboratory as purified tuberculin RT 23, using essentially the same technique. Purified tuberculin RT 32 and *N. farcinica* sensitin RS 84 (14) were dissolved in 0.15 M phosphate buffered saline (PBS) pH 7.4 at a concentration of 1 mg per ml.

Direct migration inhibition tests: Eight weeks after immunization, nine non-immunized guinea-pigs, 12 immunized with *M. tuberculosis* and 14 with *N. farcinica*, were injected intraperitoneally (i.p.) with 10 ml sterile paraffin oil. 17 of these animals (five non-immunized, five immunized with *M. tuberculosis* and seven immunized with *N. farcinica*) had been skin tested 5 weeks previously. Three days later the exudate was harvested after i.p. injection of 10 ml heparinized Hanks balanced salt solution (HBSS). The PEC were washed twice in HBSS and suspended at 8×10^7 cells per ml in TC-199 adjusted to pH 7.4 containing Hepes buffer 6.2 g per l and 10 per cent NGPS. Antigen solution for stimulations was added to achieve a concentration of 20 µg per ml. An equivalent volume of PBS was added to control suspensions. The suspensions were incubated at 37° C for 45 min in a moist chamber. 7 µl of each of the PEC suspensions were deposited in three holes in the agarose plates. The plates were incubated at 37° C for 20 hours in 5 per cent CO_2 in air in a moist chamber. The cells and the agarose were fixed in glutaraldehyde 7.5 per cent for 30 min. The agarose was removed from the dishes and the cells adhering to the bottom were rinsed with distilled water and dried. The migration areas were measured by planimetry. Migration indices (MI) were calculated as follows:

$$MI = \frac{\text{Mean migration area in antigen-stimulated cultures}}{\text{Mean migration area in PBS-stimulated cultures}}$$

Preparation of culture supernatants: Inguinal, axillary and cervical lymph nodes from six guinea-pigs immunized with *M. tuberculosis* and from seven non-immunized guinea-pigs were isolated and collected in cold HBSS. The nodes were cut into small pieces and squeezed through a # 100 stainless steel mesh. The cells were washed twice in HBSS and finally suspended in completely supplemented Eagles minimal essential medium containing 10 per cent NGPS in 12 × 100 mm plastic tubes (NUNC, Roskilde, Denmark) at a concentration of 2.4×10^7 cells per ml. PPD dissolved in PBS was added to a concentration of 20 or 50 µg per ml, or the equivalent volume of PBS was added. After incubation at 37° C in 5 per cent CO_2 in air for 24 hours, the cultures were centrifuged at 600 g for 20 min. The supernatants were kept at –20° C until use.

Testing of supernatants: PPD was added to supernatants from cultures of cells stimulated with PBS so that the concentration reached that in the cultures containing tuberculin and the same volume of PBS was added to supernatants from cells stimulated with PPD. PEC harvested from a non-immunized guinea-pig were suspended in supernatants from cells cultured with PPD and in supernatants from cells cultured with PBS to give a final concentration of 8×10^7 cells per ml. The PEC suspended in supernatants were pre-incubated for 1.5 hours at 37° C in 5 per cent CO_2 in air. Each suspension was deposited in three holes in agarose plates, and after incubation at 37° C in 5 per cent CO_2 in air for 20 hours in a moist chamber, the cells were fixed with glutaraldehyde 7 5 per cent.

The migration indices were calculated as follows:

$$MI = \frac{\text{Mean migration area of PEC in PPD supernatant}}{\text{Mean migration area of PEC in PBS supernatant}}$$

Statistical analysis: Evaluation of the results was made by ranking and the significance of differences between two samples or of paired differences was tested by the Wilcoxon test (8).

RESULTS

Skin Tests

The results of the skin tests (Fig. 1) show that all the the immunized guinea-pigs had developed hypersensitivity, since the diameters of reactions in all the immunized guinea-pigs were significantly larger than those of the non-immunized animals ($p<0.01$). Some cross reactivity was demonstrated, since the diameters of reactions to the heterologous antigen in the immunized animals were significantly larger than those in the non-immunized animals ($p<0.01$). In each of the immunized guinea-pigs, the homologous reactions were significantly larger than the heterologous reactions ($p<0.01$). In the non-immunized animals no systematic differences in response to the two preparations were obtained.

The reactions of the immunized animals increased when the dose of tuberculin or *N. farcinica* sensitin was increased tenfold ($p<0.01$). The reactions of the non-immunized animals did not increase with a tenfold increase in dose of the two preparations ($p>0.1$).

Direct Inhibition of Cell Migration

The results are presented in Fig. 2. The migration indices of cells originating from 12 guinea-pigs immunized with *M. tuberculosis* and stimulated with tuberculin varied from 0.35 to 0.56. These values differ significantly from those obtained with cells originating from nine non-immunized guinea-pigs which were stimulated with tuberculin, range 0.82–1.03 ($p<0.01$). The migration indices of cells originating from 14 animals immunized with *N. farcinica* and stimulated with *N. farcinica* sensitin varied from 0.38 to 0.95. These values differ significantly from those obtained with cells from nine non-immunized animals stimulated with *N. farcinica* sensitin, range 0.72–0.98 ($p<0.01$).

The migration indices of cells from animals immunized with *M. tuberculosis* in cultures stimulated with *N. farcinica* sensitin varied from 0.69 to 0.95. These indices did not differ from those obtained similarly in non-immunized guinea-pigs, range 0 72–0.98 ($0.05<p<0.10$). The migration indices of cells from guinea-pigs immunized with *N. farcinica* in cultures stimulated with tuberculin varied from 0.70 to 0.95. These values differed from those obtained similarly in

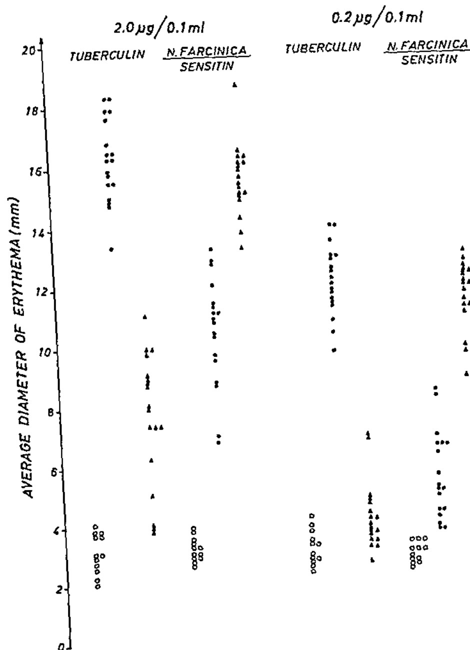


Fig. 1. Average diameters of erythema (in mm) elicited by tuberculin or *N. farcinica* sensitin in non-immunized guinea-pigs ○, guinea-pigs immunized with *M. tuberculosis* ● or *N. farcinica* ▲.

non-immunized animals, range 0.82–1.03 ($p = 0.05$). In each of the immunized guinea-pigs the migration inhibition of cells was significantly larger when the cells were stimulated with the homologous sensitin than with the heterologous sensitin ($p<0.01$). In the non-immunized guinea-pigs a significant difference in migration inhibition was obtained between tuberculin and *N. farcinica* sensitin as stimulator of the cells ($p = 0.05$). The migration indices of cells from guinea-pigs which had been skin tested prior to the

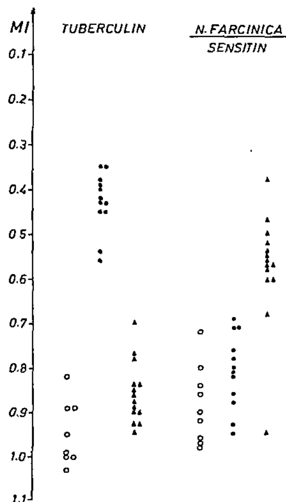


Fig 2. Migration indices of PEC from non-immunized guinea-pigs ○, guinea-pigs immunized with *M. tuberculosis* ● or *N. farcinica* ▲. The cultures were stimulated with tuberculin or *N. farcinica* sensitin

in vitro assay did not differ systematically from those of cells from guinea-pigs which had not been skin tested (p>0.10).

Inhibition of Cell Migration with Culture Supernatants

The results are presented in Fig. 3. The supernatants of lymph node cells from guinea-pigs immunized with *M. tuberculosis* and stimulated with PPD 20 µg per ml were able to inhibit significantly (p<0.01) the migration of PEC from non-immunized guiena-pigs. The migration indices varied from 0.53 to 0.78. Stimulation with a larger dose of PPD, 50 µg per ml, apparently had a slightly stronger effect, range 0.42–0.76. However, this difference was not significant. Migration indices of PEC from non-immunized guinea-pigs exposed to supernatants from lymph node cells of non-immunized guinea-pigs stimulated with 20 or 50 µg tuberculin did not differ significantly (p>0.10).

DISCUSSION

The present study confirms the results of *Carpenter et al.* (2), who showed that inhibition of the migration of PEC from immu-

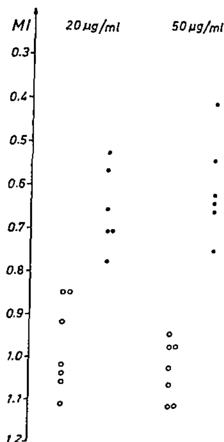


Fig. 3. Migration indices of non-immunized guinea-pig PEC exposed to supernatants from cultures of lymph node cells from non-immunized guinea-pigs ○ and guinea-pigs immunized with *M. tuberculosis* ●. The cultures were stimulated with 20 µg or 50 µg tuberculin per ml.

nized guinea-pigs can be demonstrated by the agarose plate technique. The doses of antigen used by us are the same as those used commonly in leucocyte migration studies. The statistical methods used in our study do not require that the migration indices are normally distributed.

The specificity of the inhibition of cell migration has been demonstrated by *Darlington & Scherago* (5) with tuberculin and brucellergen in cultures of peripheral blood leucocytes from *Brucella*-infected guinea-pigs. Later, using the capillary tube technique, *David et al.* (6) showed that the inhibition of migration of PEC from guinea-pigs immunized with diphtheria toxoid and ovalbumin is specific. In the present study, the inhibition of migration obtained with PPD and *N. farcinica* sensitin was found to be specific. The slight inhibition of cells from guinea-pigs immunized with *N. farcinica* and stimulated with tuberculin is undoubtedly a manifestation of a slight cross reactivity between the two antigens. This cross reactivity is more clearly demonstrated by the skin tests, which may indicate that the sensitivity of the agarose plate technique is lower than that of the skin test technique. A lower sensitivity of the agarose plate technique might explain why no inhibition of migration could be demonstrated (MI = 0.95) with the cells from one guinea-pig immunized with *N. farcinica*, despite the fact that hypersensitivity was demonstrated by the skin tests.

The present study shows that culture supernatants from lymph node cells of immunized guinea-pigs were able to inhibit the migration of PEC from non-immunized guinea-pigs in the agarose plate technique. As pointed out by *Clausen* (4), fewer cells from immunized individuals are needed to produce supernatants with MIF activity than are needed in the direct migration test. Another advantage is that several supernatants can be tested for MIF activity on the same indicator cell population.

Injection of antigen for skin testing may alter the degree of immunity of the injected individuals to that antigen. Thus, it has been demonstrated in tuberculin-positive humans that the blast transformation response to PPD *in vitro* increases for several weeks following the tuberculin skin test (10). In our study on a limited number of guinea-pigs, skin testing 5 weeks prior to the *in vitro* testing had no influence on the antigen-dependant migration inhibition of the animal cells.

The technique used in this study is essentially the same as that described by *Clausen* (3, 4). However, we have found that a smaller number of migrating guinea-pig PEC (8×10^7 per ml) compared to the number of human blood leucocytes (2.2×10^8 per ml) produced larger migration areas (unpublished experiments).

In the present study, the specificity and applicability of the agarose plate technique in guinea-pigs have been elucidated. Further studies in guinea-pigs differing with respect to the degree of hypersensitivity are required to elucidate the sensitivity of the method.

REFERENCES

1. *Bloom, B. B. & Bennett, B.:* Mechanisms of a reaction *in vitro* associated with delayed-type hypersensitivity. Science *153:* 80–82, 1966.
2. *Carpenter, R. R., Batsales, P. B. & Gancher, R. P.:* Antigen-induced inhibition of cell migration in agar gel, plasma clot and liquid media. J. reticul. Soc. *5:* 472–483, 1968.
3. *Clausen, J. E.:* Tuberculin-induced migration of human peripheral leucocytes in agarose medium. Acta allerg. (Kbh.) *26:* 56–80, 1971.
4. *Clausen, J. E.:* Migration inhibitory effect of cell-free supernatants from tuberculin-stimulated cultures of human mononuclear leucocytes demonstrated by two-step MIF agarose assay. J. Immunol. *110:* 546–551, 1973.
5. *Darlington, R. W. & Scherago, M.:* The *in vitro* sensitivity to brucellergen of leucocytes from guinea-pigs experimentally infected with *Brucella abortus*. J. infect. Dis. *106:* 106–110, 1960.
6. *David, J. R., Al-Askari, S., Lawrence, H. S. & Thomas, L.:* Delayed hypersensitivity *in vitro*. I. The specificity of inhibition of cell migration by antigens. J. Immunol. *93:* 264–273, 1964.
7. *David, J. R.:* Delayed hypersensitivity *in vitro:* Its mediation by cell-free substances formed by lymphoid cell-antigen interaction. Proc. nat. Acad. Sci. (Wash.) *56:* 72–77, 1966.

8. *Diem, J.* (Ed.): Documenta Geigy, Scientific Tables. 6. ed. J. R. Geigy S. A., Basel 1962, p. 191–192.
9. *George, M. & Vaughan, J. H.: In vitro* cell *migration as a model for delayed hypersensitivity.* Proc. Soc. exp. Biol. (N.Y.) *111:* 514–521, 1962.
10. *Hughes, N. R.:* Tuberculin sensitive lymphocytes in human peripheral blood following Mantoux test. Aust. J. exp. Biol. med. Sci. *46:* 619–629, 1968.
11. *Kim. H. K., Magnusson, M. & Bentzon, M. W.:* Tuberculin production 2. Comparison between amount of tuberculin obtained from the culture filtrate and by extraction of tubercle bacilli. Acta path. microbiol. scand. *58:* 501–510, 1963.
12. *Magnusson, M., Guld, J., Magnus, K. & Waaler, H.:* Diluents for stabilization of tuberculin. Bull. Wld Hlth Org. *19:* 799–828, 1958.
13. *Magnusson, M. & Bentzon, M. W.:* Preparation of purified tuberculin RT 23. Bull. Wld Hlth Org. *19:* 829–843, 1958.
14. *Magnusson, M.:* Specificity of sensitins. III. Further studies in guinea-pigs with sensitin of various species of *Mycobacterium* and *Nocardia.* Amer. Rev. resp. Dis. *86:* 395–404, 1962.
15. *Magnusson, M. & Mariat, F.:* Delineation of *Nocardia farcinica* by delayed type skin reactions on guinea-pigs. J. gen. Microbiol. *51:* 151–158, 1968.
16. *Rich, R. A. & Lewis, M. R.:* Mechanism of allergy in tuberculosis. Proc. Soc. exp. Biol. (N.Y.) *25:* 596–598, 1928.

Acta path. microbiol. scand. Sect. C, 83: 59–73, 1975

FUNCTIONAL ALTERATIONS INDUCED BY CHLORPROMAZINE IN MONONUCLEAR BLOOD CELLS CULTURED *IN VITRO*

ARNE ØDEGAARD

Trondheim Central Hospital, Department of Medicine, Section for Haematology and Immunology, Trondheim, Norway

Ødegaard, A.: Functional alterations induced by chlorpromazine in mononuclear blood cells cultured *in vitro*. Acta path. microbiol. scand. Sect. C, 83: 59–73, 1975.

The effect of Chlorpromazine (CPZ), a membrane active drug, on the morphology and function of blood monocytes and lymphocytes cultured *in vitro*, was studied. The experiments indicate that CPZ exhibits a strong and dose dependent effect on cell membrane functions like cell adhesiveness and phagocytosis of radiolabelled Candida particles. A biphasic effect was found on the attachment of cells at culture start and on the attachment/engulfment of particles in the cultured cells. Low drug concentrations were found to increase adhesiveness while higher concentrations caused cell lysis. Chlorpromazine in lytic concentrations decreased the intracellular digestion of Candida particles. Small drug concentrations did not appear to influence the digestion of particles as observed morphologically, but a decreased release of metabolized ^{125}Iodine to the medium was found, suggesting a stabilization of the cell membrane, with reduced transfer of the digested material to the medium.

Key words: Mononuclear blood cells; alterations; chlorpromazine.

A. Ødegaard, Department of Medicine, Trondheim Central Hospital, 7000 Trondheim, Norway.

Received 13.viii.74 Accepted 22.viii.74

There is experimental evidence that Chlorpromazine, a tranquillizing drug, excerts its effect upon the nerve cell membrane mainly by, changing its physical and biochemical properties (6, 16). Side effects and toxic manifestations during CPZ treatment have been observed, some of them being Parkinsonism, jaundice and reduced resistance to infections (5). Infection resistance involves both immunological and inflammatory reactions. It is well documented that the thymus-derived lymphocyte is essential in cell-mediated immunological reactions and that the mononuclear phagocyte serves as a scavenger cell in chronic inflammatory reactions. The aim of the present work was to study the effect of Chlorpromazine on these cells, using standardized *in vitro* techniques for the registration of lymphocyte and monocyte functions.

MATERIALS AND METHODS

The general methods for culturing of human monocytes and for testing of phagocytosis have been reported previously (20 21).

^{51}Cr-labelling of Mononuclear cells (14)

The separated mononuclear cells were suspended in Hanks balanced salt solution (Hanks BSS)

supplemented with Tris-HCl. The cell suspension adjusted to 5 × 10^6 cells per ml was incubated for 30 minutes at 37° C with 10 µCi ^{51}Cr (carrier free, Kjeller, Norway), per ml cell suspension. After radiolabelling the cells were washed twice in excess of Hanks BSS before culturing.

^{125}I-labelling of Candida

Candida albicans particles (Dept. of microbiology, Trondheim Central Hospital) were heat killed at 60° C for 2 hours and labelled with ^{125}I by means of electrolysis (19).

Chlorpromazine

Chlorpromazini chloridum (Kindly supplied by Dumex) was dissolved in and diluted with saline and steril-filtered (Millipore—pore size 0.2 µm) before use.

Cell Culture

Mononuclear blood cells were separated from defibrinated venous blood of healthy adults by a method described by *Bøyum* (1). The cells were cultured as monolayers in plastic Petri dishes or on glass coverslips in Parkers medium 199 supplemented with l-glutamine and 20 per cent homologous AB serum. Streptomycin and penicillin were added at 100 µg/ml and 100 units/ml, respectively. The cell cultures were incubated in a National CO_2-incubator at 37° C with 5 per cent CO_2 in air and with 100 per cent humidity.

Test Methods

Two test models for the registration of drug effect on the cells were established.

Test model I (Fig. 1) shows the registration procedure of different functions of young mononuclear cells after exposure to the drug. In this study the drug was added to the medium immediately after delivery of the cell suspensions to the culture dishes. The cultured cells were exposed to the drug during a 90 minutes incubation period. The medium containing the non-adherent cells and the drug was then removed, the cells at the bottom of the Petri dishes were flushed with medium to remove loosely attached cells, and 2.5 ml fresh complete medium without drug was added to each culture dish.

Cellular Adhesiveness

The numbers of adhesive cells were estimated as the differences between the numbers of leucocytes added to the culture dishes at culture start and the numbers of cells removed after 90 minutes' incubation. The cell numbers were counted in an electronic particle counter (Coulter, type Fn).

Preparation of Rosette-forming Lymphocytes

The method was based on that described by *Munro et al.* (9). The non-adhesive cells removed after 90 minutes' incubation, consisting mainly of lymphocytes, were washed twice in Hanks BSS. The final suspension was adjusted to contain 5 × 10^6 cells per ml. Equal volumes (0.6 ml) of the lymphocyte suspension and a 0.5 per cent suspension of sheep red blood cells were added to 0.6 ml Hanks BSS containing 20 per cent absorbed guinea-pig serum. (Guinea-pig serum was prepared by absorbing with equal volumes of washed human and sheep red blood cells). The suspension was then spun down at 200 × *g* for 5 minutes followed by incubation for 30 minutes at 4° C. After resuspension, the number of rosette-forming lymphocytes per 1000 cells was counted in a microchamber using phase-contrast microscopy.

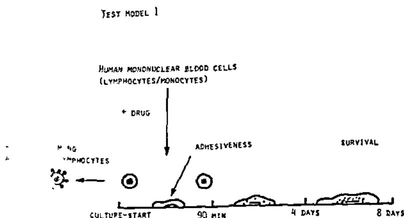


Fig gistration of different functions of young mononuclear cells.

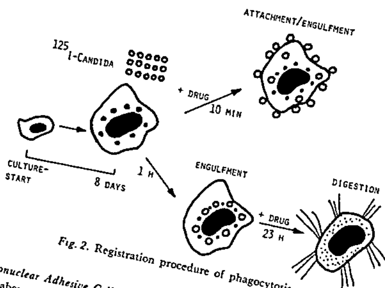


Fig. 2. Registration procedure of phagocytosis.

Survival of Mononuclear Adhesive Cells

As described above, the adhesive cells were cultured for 4 days and then counted directly, using a Reichert inverted microscope with phase contrast.

Registration of phagocytosis

Test model II (Fig 2) shows the registration procedure of phagocytosis. Monocytes cultured on coverslips were tested for phagocytosis after different periods of culturing (4 days and 8 days). Engulfment and digestion were studied in separate experiments.

In the engulfment experiments, the cells were incubated with a constant number of Candida and Chlorpromazine in varying concentrations. The cells were harvested after a period of phagocytosis of 10 minutes The coverslips were washed 6 times in Hanks BSS and the radioactivity on the coverslips registered in a Wallac GM gamma radiation counter. The ratio between the number of Candida particles added and the number of adhesive cells was usually about 20·1

In the digestion experiments a constant number of Candida was added to 8 days old coverslip cultures After a further incubation for 10 minutes the coverslips were washed 3 times and placed in a new Petri dish containing fresh culture medium. Chlorpromazine in varying concentrations was added to the culture medium one hour later. The cultures were harvested after 24 hours' incubation. The medium was collected, centrifuged at 2000 × g for 5 minutes and the radioactivity in the supernatant and in the sediment was measured separately. The radioactivity on the coverslips was registered as a measure of the amount of Candida left.

Cell Morphology

The cells were cultured on coverslips in plastic Petri dishes. On day 8 of culturing the coverslips were removed, rinsed in Hanks BSS and inverted on microchambers as described previously (21). The chambers were filled with medium containing Chlorpromazine in varying concentrations, sealed with wax and studied in a Leitz Laborlux phase contrast microscope with automatic equipment for photography. The chambers could be incubated at 37° C for several hours. In the digestion experiments, at the time of harvesting, some of the coverslips were inverted on microchambers and observed morphologically.

RESULTS

Cellular Adhesiveness

Two different methods have been used to investigate the effect of Chlorpromazine on cellular adhesiveness. The data given in Table 1 are based upon an objective registration by counting cells in an electronic particle counter, the data given in Table 2 are based upon direct cell counting in a phase contrast microscope.

TABLE 1. *Effect of Chlorpromazine on the Adhesiveness of Blood Mononuclear Cells after 90 Minutes Exposure to the Drug*

Culture series no.	Cell numbers $\times 10^6$, added	Concentrations of CPZ, Mol 5×10^{-4}	10^{-4}	10^{-5}	10^{-6}	Control without CPZ
105	8.1	*	2.9	–	–	2.7
106	10.8	*	4.1	–	–	3.7
107	4.5	*	0.6	0.5	0.5	0.4
108	7.6	*	2.2	1.6	1.4	1.1
109	6.5	*	1.9	1.8	1.5	1.5
110	6.3	–	–	1.0	0.9	0.5
111	7.0	–	3.4	3.1	3.1	2.9

Numbers of adhesive cells per culture dish $\times 10^6$, estimated as the difference between the numbers of cells added to the culture dishes and the numbers of cells removed after 90 minutes' incubation.

– not tested.

* Microscopic examination of removed cells after 90 minutes' incubation showed cell lysis and aggregation. Microscopic examination of the Petri dishes showed no adherent cells at the given concentration of CPZ.

TABLE 2. *Effect of Chlorpromazine on Survival of Blood Mononuclear Phagocytes Cultured in Vitro*

Culture series no.	Concentrations of CPZ, Mol 5×10^{-4}	10^{-4}	10^{-5}	10^{-6}	Control without CPZ
105	0	8.8	–	–	7.2
106	0	18.9	–	–	12.7
107	0	4.9	4.7	4.1	3.8
108	0	11.2	11.1	10.1	9.7
109	0	17.5	15.0	11.4	9.2
110	–	–	5.9	5.8	4.6
111	–	12.2	11.5	10.7	10.0

The cells were exposed to the drug for 90 minutes at culture start and the numbers of adhesive phagocytes counted after 4 days' culturing. The values given are the means of the cell numbers $\times 10^4$ in triplicate culture dishes.

– not tested.

Chlorpromazine in a final concentration between 10^{-6} and 10^{-4} Mol increased the adhesiveness of mononuclear blood cells to plastic surfaces at culture start (Fig. 3, Table 1). The cell numbers given in Table 2 were obtained on the fourth day of culturing indicating that the drug concentrations which stimulated cellular adhesiveness at culture start, did not cause reduced survival at this period of time (Fig. 4, Table 2). The stimulating effect was dose dependent. In a concentration of 5×10^{-4} Mol, CPZ caused cell lysis, detachment from the surface and aggregation. At this concentration of the drug, a marked release of ^{51}Cr from the cells was observed (Fig. 4), a finding which indicates an increased permeability of the cell membrane.

Rosette Forming Ability of Lymphocytes

After a pre-incubation period of 90 minutes with concentrations of CPZ varying between 10^{-6} and 10^{-4} Mol, lymphocytes showed no marked alteration in their ability to form rosettes with sheep red blood cells (Fig. 5, Table 3). At a drug concentration of 5×10^{-4} Mol, the mononuclear cells were aggregated and no rosettes were observed.

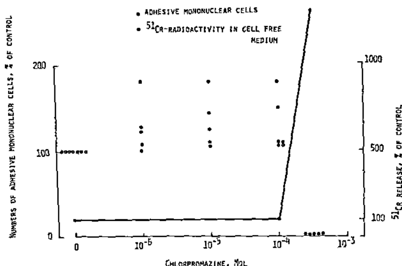


Fig. 3. The numbers of adhesive mononuclear blood cells in plastic Petri dishes after an incubation period of 90 minutes with different concentrations of CPZ in the culture medium. The numbers of adhesive cells were estimated as the difference between the number of cells added to the culture dishes and the numbers of cells removed after 90 minutes' incubation. The values given are the means of the cell numbers in triplicate culture dishes and are expressed as percentages of the control values without CPZ added. The cells had been labelled with ^{51}Cr. Release of ^{51}Cr to the culture medium, expressed as percentages of the control values, is used as a measure of cell damage.

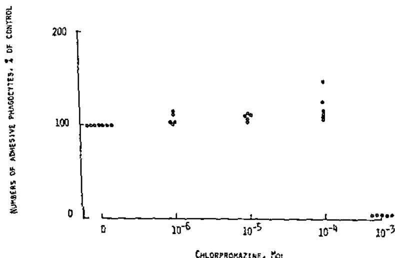


Fig. 4. The numbers of adhesive mononuclear phagocytes after an incubation period of 90 minutes with different concentrations of CPZ in the culture medium. The numbers of adhesive cells were counted after 4 days' culturing. The values given are the means of the cell numbers in triplicate culture dishes and are expressed as percentages of the control samples without CPZ added.

Attachment/Engulfment of Radiolabelled Heat-killed Candida

Chlorpromazine in concentrations between 10^{-5} and 10^{-4} Mol slightly stimulated the attachment/engulfment of yeast particles in 4 and 8 days old mononuclear phagocytes cultured *in vitro* (Figs. 6 and 7, Tables 4 and 5). At drug concentrations exceeding 10^{-4}

TABLE 1. *Effect of Chlorpromazine on the Adhesiveness of Blood Mononuclear Cells after 90 Minutes' Exposure to the Drug*

Culture series no.	Cell numbers $\times 10^6$, added	Concentrations of CPZ, Mol 5×10^{-4}	10^{-4}	10^{-5}	10^{-6}	Control without CPZ
105	8.1	*	2.9	–	–	2.7
106	10.8	*	4.1	–	–	3.7
107	4.5	*	0.6	0.5	0.5	0.4
108	7.6	*	2.2	1.6	1.4	1.1
109	6.5	*	1.9	1.8	1.5	1.5
110	6.3	–	–	1.0	0.9	0.5
111	7.0	–	3.4	3.1	3.1	2.9

Numbers of adhesive cells per culture dish $\times 10^6$, estimated as the difference between the numbers of cells added to the culture dishes and the numbers of cells removed after 90 minutes' incubation.
– not tested.
* Microscopic examination of removed cells after 90 minutes' incubation showed cell lysis and aggregation. Microscopic examination of the Petri dishes showed no adherent cells at the given concentration of CPZ.

TABLE 2. *Effect of Chlorpromazine on Survival of Blood Mononuclear Phagocytes Cultured* in Vitro

Culture series no.	Concentrations of CPZ, Mol 5×10^{-4}	10^{-4}	10^{-5}	10^{-6}	Control without CPZ
105	0	8.8	–	–	7.2
106	0	18.9	–	–	12.7
107	0	4.9	4.7	4.1	3.8
108	0	11.2	11.1	10.1	9.7
109	0	17.5	15.0	11.4	9.2
110	–	–	5.9	5.8	4.6
111	–	12.2	11.5	10.7	10.0

The cells were exposed to the drug for 90 minutes at culture start and the numbers of adhesive phagocytes counted after 4 days' culturing. The values given are the means of the cell numbers $\times 10^4$ in triplicate culture dishes.
– not tested.

Chlorpromazine in a final concentration between 10^{-6} and 10^{-4} Mol increased the adhesiveness of mononuclear blood cells to plastic surfaces at culture start (Fig. 3, Table 1). The cell numbers given in Table 2 were obtained on the fourth day of culturing indicating that the drug concentrations which stimulated cellular adhesiveness at culture start, did not cause reduced survival at this period of time (Fig 4, Table 2). The stimulating effect was dose dependent. In a concentration of 5×10^{-4} Mol, CPZ caused cell lysis, detachment from the surface and aggregation. At this concentration of the drug, a marked release of ^{51}Cr from the cells was observed (Fig. 4), a finding which indicates an increased permeability of the cell membrane.

Rosette Forming Ability of Lymphocytes

After a pre-incubation period of 90 minutes with concentrations of CPZ varying between 10^{-6} and 10^{-4} Mol, lymphocytes showed no marked alteration in their ability to form rosettes with sheep red blood cells (Fig. 5, Table 3). At a drug concentration of 5×10^{-4} Mol, the mononuclear cells were aggregated and no rosettes were observed.

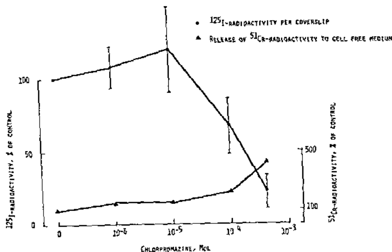


Fig. 6. Effect of CPZ on the attachment/engulfment of ^{125}I-labelled heat-killed Candida particles in blood mononuclear phagocytes cultured for 4 days on coverslips. Mean values of quadruplicate coverslips in 11 experiments are given, expressed as percentages of the control values without CPZ added. Standard deviations are marked as bars. Release of ^{51}Cr from radiolabelled cells incubated with different concentrations of CPZ is also given as percentages of the values without CPZ.

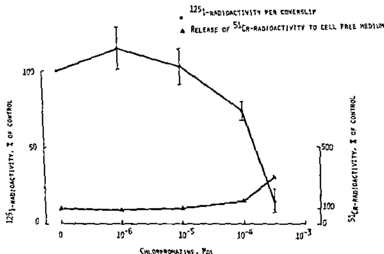


Fig. 7. Effect of CPZ on the attachment/engulfment of ^{125}I-labelled heat-killed Candida particles in blood mononuclear phagocytes cultured for 8 days on coverslips. Mean values of quadruplicate coverslips in 9 experiments are given, expressed as percentages of the control values without CPZ added. Standard deviations are marked as bars. Release of ^{51}Cr from radiolabelled cells incubated with different concentrations of CPZ is also given as percentages of the values without CPZ.

added radioactivity found in the cell free medium at harvesting. As shown previously (20), radiolabelled Candida particles will passively release approximately 12 per cent radioactivity when incubated for 1 to 3 days. This passive release was not corrected for in the values listed in Fig. 8 and Table 6.

Chlorpromazine in a final concentration between 10^{-5} and 5×10^{-4} Mol reduced the release of ^{125}Iodine to cell free medium. At harvesting, the cells exposed to different concentrations of CPZ were observed micro-

TABLE 4. *Effect of Different Concentrations of Chlorpromazine on the Attachment/Engulfment of Radiolabelled Heat-killed Candida in 4 Days Old Blood Mononuclear Phagocytes Cultured* in Vitro *on Glass Coverslips*

Culture series no.	Concentrations of CPZ, Mol				Control without CPZ
	5×10^{-4}	10^{-4}	10^{-5}	10^{-6}	
105	7	20	–	–	34
106	–	34	45	–	50
107	2	–	29	–	29
108	1	8	13	–	11
109	3	16	19	–	19
110	3	6	11	–	8
111	1	9	10	–	11
112	2	14	17	19	22
113	7	73	116	136	117
114	2	15	28	31	29
115	5	14	19	17	15

The figures listed are the means of the radioactivity (ct/min $\times 10^2$) in quadruplicate coverslips.
– not tested.

TABLE 5. *Effect of Different Concentrations of Chlorpromazine on the Attachment/Engulfment of Radiolabelled Heat-killed Candida in 8 Days Old Blood Mononuclear Phagocytes Cultured* in Vitro *on Glass Coverslips*

Culture series no.	Concentrations of CPZ, Mol				Control without CPZ
	5×10^{-4}	10^{-4}	10^{-5}	10^{-6}	
105	4	14	16	–	17
106	15	21	–	–	54
107	5	20	50	–	33
108	4	66	96	138	111
109	25	105	233	156	139
110	27	–	–	54	62
111	9	29	37	35	30
112	3	9	20	18	17
113	8	20	27	28	29

The figures listed are the means of the radioactivity (ct/min $\times 10^2$) in quadruplicate coverslips.
– not tested

scopically. At a drug concentrations of 5×10^{-4} Mol, the cells were detached from the coverslips At 10^{-4} Mol, however, a few cells remained at the coverslips. These cells showed disintegrated cytoplasm and undigested Candida particles in the central cytoplasm (Fig. 9 c)

In the cultures incubated with Chlorpromazine in a concentration of 10^{-6} Mol and in the control cultures without CPZ added, the cells showed increased cytoplasmic granulation and no intact particles left in the cells could be observed (Fig. 9 e). Thread-like membrane projections could be observed. The cells exposed to CPZ in a concentration of 10^{-5} Mol appeared to be able to digest the engulfed yeast particles, as observed morphologically (Fig. 9 d). However, these cells lacked the membrane projections which were seen in the control cultures at harvesting. At this concentration of CPZ a decreased release of ^{125}Iodine was found in the cell free me-

TABLE 6. *Effect of Different Concentrations of Chlorpromazine on the Digestion of Engulfed Radio-labelled Heat-killed Candida in 8 Days Old Blood Mononuclear Phagocytes Cultured* in Vitro *on Glass Coverslips*

Concentrations of CPZ, Mol	Radioactivity coverslips ct/min × 10^2	%*	sediment ct/min × 10^2	%*	cell free medium ct/min × 10^2	%*	Total per culture dish ct/min × 10^2
5 × 10^{-4}	12 12	18	92	69	17	13	133
	10 12	17	87	69	18	14	127
10^{-4}	12 9	26	42	53	17	21	80
	11 7	23	42	55	17	21	77
10^{-5}	15 23	43	4	4	47	53	89
	12 13	42	2	4	33	55	60
10^{-6}	15 14	27	3	3	76	70	108
	7 7	26	9	7	30	57	53
Control without CPZ	11 11	22	5	5	73	73	100
	12 17	31	4	4	62	65	95

The figures listed are the results from one typical digestion experiment performed in duplicate culture dishes.
* per cent of total radioactivity per culture dish.

dium, which may be due to a stabilization of the cell membrane with reduced transfer of radioactivity. This explanation was supported by the fact that no increase in the release of ^{51}Cr was found at this concentration of CPZ, compared to the control cultures.

The high percentage of ^{125}Iodine radioactivity found in the sediment from the cultures incubated with CPZ at a final concentration between 10^{-4} and 5 × 10^{-4} Mol reflects undigested Candida in detached or lysed cells. Microscopical examination of the sediment showed cell debris, free Candida particles and a great amount of detached cells containing undigested Candida.

DISCUSSION

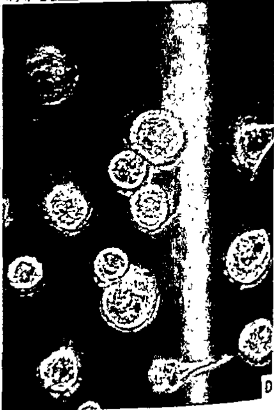
The *in vitro* effect of Chlorpromazine on various cells has been extensively investigated. It has been documented that the drug exerts its effect mainly on the cell surface membrane and on intracellular membranes (16). The most predominant biochemical characteristic feature of CPZ is described as a dose dependent, biphasic effect on membrane permeability (16). The drug has been tested for its effect on hypotonic haemolysis of human erythrocytes, and a biphasic pattern of stabilization and lysis has been found (16).

Decreased resistance to infection has been

5*


A

B

C

D

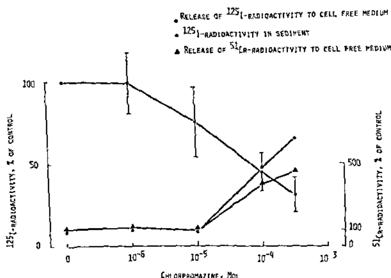


Fig. 8. Effect of CPZ on digestion of ^{125}I-labelled heat-killed Candida particles in blood mononuclear phagocytes cultured for 8 days on coverslips. The values are the means of the results from 9 experimnts performed in duplicate culture dishes and registered 23 hours following the engulfment phase. Standard deviations are marked as bars. Release of ^{51}Cr from radiolabelled cells incubated with different concentrations of CPZ is also given as percentages of the values without CPZ.

Fig. 9. Effect of CPZ on digestion of heat-killed *Candida albicans* in blood mononuclear phagocytes during an incubation period of 24 hours.

A Yeast particles adherent to the cell surface after incubation with the yeast for 10 min

B Phagocytic cells after incubation for one hour. The yeast particles are engulfed and located in the central cytoplasm.

C After 23 hours' incubation with CPZ in a concentration of 10^{-4} Mol. The cell cytoplasm is disintegrated and contains indigested Candida particles.

D After 23 hours' incubation with exposure to CPZ in a concentration of 10^{-5} Mol. The cell cytoplasm is granular in appearance. No membrane projections are visible.

E After 23 hours' incubation without drug. No intact Candida particles can be seen and the cytoplasm is granular. Thread-like projections of the cell membrane can be seen.

Phase-contrast × 1480.



observed after CPZ treatment (5). The present investigations suggest that this decreased resistance may be due to an effect of the drug on lymphocytes and monocytes. The thymus-derived lymphocytes are essential in cell-mediated immunological reaction. As tissue macrophages, the blood monocytes serve as scavenger cells in chronic inflammatory reactions and appear to be of importance in the immunological reaction to antigens.

Cellular adhesion and phagocytosis are biological phenomena depending on an intact cell membrane. The effect of CPZ on phagocytosis is therefore reasonable. CPZ has been found to inhibit phagocytosis in human neutrophilic granulocytes. *Kvarstein & Stormorken* who studied the phagocytosis of polysterene latex particles, found a dose dependent inhibition on the engulfment of particles and oxygen consumption in human neutrophils at a concentration of CPZ between 1.4×10^{-4} and 1.1×10^{-3} Mol (7). *Ruutu* using viable bacteria found decreased engulfment of bacteria in human neutrophilic granulocytes at a CPZ concentration between 10^{-4} and 10^{-3} Mol and a slightly increased engulfment of bacteria at 10^{-7} Mol. CPZ at 10^{-4} reduced the intracellular killing of viable engulfed bacteria (12). In the present investigation, similar concentrations of CPZ reduced phagocytosis of radiolabelled heat killed Candida in human monocytes cultured on coverslips.

Human monocytes are adhesive cells and will stick to surfaces when cultured *in vitro* (21). The ability of the cell membrane to adhere to surfaces is a vital function of the cell, depending upon an intact cell metabolism. Adhesiveness and other functions of the cell membrane like engulfment and mobility by pseudopodial processes play an important role in the function of inflammatory cells. The majority of lymphocytes are non-adhesive cells. After a short incubation period at culture-start it is possible to separate the two different types of mononuclear blood cells because of the difference in adhesiveness and to study some of their functions separately.

The function of lymphocytes was studied in the rosette formation test which measures the ability of lymphocytes to form spontaneous rosettes with sheep red blood cells. At lytic concentrations of CPZ, the lymphocytes were aggregated and lysed and no rosette forming cells were found. . No reduction in the numbers of rosettes was found at lower concentrations of the drug.

The function of monocytes and macrophages was studied more extensively. An attempt was made to correlate the effect of the drug on various steps of phagocytosis with the effect on cell morphology, cell adhesiveness and membrane permeability as measured by the release of ^{51}Cr from the cultured cells and the transfer of metabolized ^{125}Iodine from the cells to the medium.

The fact that non-lytic concentrations of CPZ slightly increased cell adhesion and attachment of particles to the cells indicates that these two phenomena reflect the same membrane function. The morphological studies of the living cells in microchambers showed an initial invagination of the cell membrane at drug concentrations that increased cell adhesion and attachment/engulfment of particles (Figs. 10 a, b, c). It is tempting to suggest that folding of the cell membrane may increase the cell surface area available for adhesion. The observation is also in accordance with the finding of *Seeman et al.* that CPZ in antihaemolytic concentrations expanded the membrane area of erythrocytes (17).

In lytic concentrations of CPZ (above 10^{-4} Mol) a marked release of ^{51}Cr from the cells was found and the cells showed signs of extensive cell damage with fragmentation and vacuolisation of the cytoplasm, as observed morphologically. Several mechanisms by which CPZ can induce lysis of cells have been suggested. According to *Santos-Martinez et al.* (15) the lytic effect on red blood cells may be due either to an increase in the permeability of the cell membrane to water or to an increase in the macromolecular permeability. Various morphological alterations in blood cells, including loss of pseudopodial formation, cytoplasmic vacuolisation

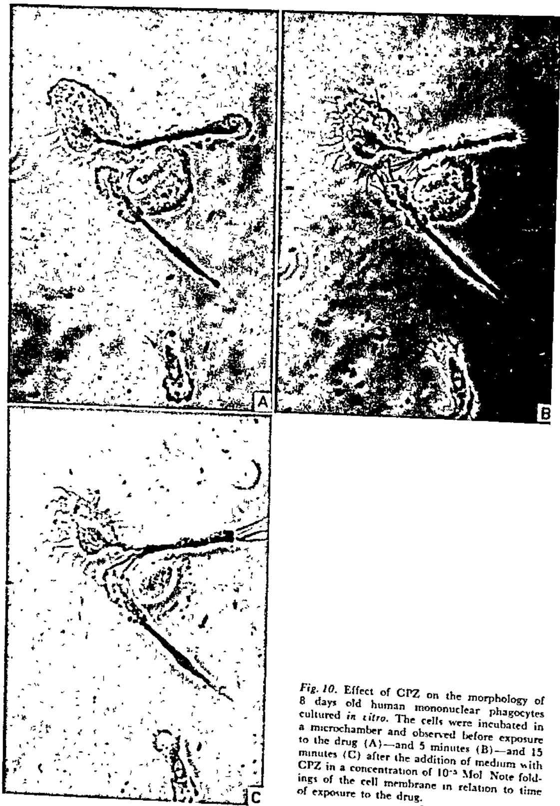


Fig. 10. Effect of CPZ on the morphology of 8 days old human mononuclear phagocytes cultured *in vitro*. The cells were incubated in a microchamber and observed before exposure to the drug (A)—and 5 minutes (B)—and 15 minutes (C) after the addition of medium with CPZ in a concentration of 10^{-5} Mol. Note foldings of the cell membrane in relation to time of exposure to the drug.

Phase-contrast × 1480.

and ultrastructural defects in the cell membrane have been demonstrated by other workers (10, 13).

Chlorpromazine in a concentration of 10^{-6} or 10^{-5} Mol did not appear to influence the intracellular digestion of particles as observed morphologically. At 10^{-5} Mol a reduced release of ^{125}Iodine to the cell free medium was found, indicating a stabilisation of the cell membrane with reduced transfer of ^{125}Iodine to the medium. Many authors have demonstrated that CPZ within the above mentioned range of concentrations is an effective inhibitor of active and passive transport of different molecules through biological membranes (4, 8). *Landmark* & *Øye* (8) demonstrated that CPZ decreased the loss of K^+ from erythrocytes and isolated rat heart cells. They concluded that the drug effect could not be due to a direct inhibition of the active K^+ transport mechanism, but was secondary to more general alterations in the physical properties of the cell membrane. *Spirtes* & *Guth* found that CPZ could stabilize the lysosome fraction of rat liver cells with up to 50 per cent inhibition of the leakage of acid phosphatase (18).

Lysosomes which have completed their digestive function and have become filled with debris are called residual bodies. There is evidence that residual bodies may fuse with the cell membrane and extrude their contents from the cells by a process of reversed pinocytosis called exoplasmosis (11). The observed membrane alterations described in this study, possibly reflect extruded material from the cells and could be a morphological counterpart to exoplasmosis. However, until the chemical and ultrastructural characteristics of this material have been further analysed, it is impossible to conclude what the membrane alterations really reflects. The observation that Chlorpromazine could inhibit the formation of these membrane projections indicate that the mechanism is dependent upon a normal function of the cell surface membrane

Human monocytes cultured *in vitro* improve their digestive capacities concomitant with an increase in the phase-dense granules locates in the perinuclear region of the cells (21). These granules have been shown to be lysosomes containing hydrolytic enzymes (2). Lytic concentrations of CPZ has been found to increase the permeability of lysosomes in bone marrow cells, resulting in a release of enzymes to the periphery of cell cytoplasm, as demonstrated by *Fand* (3). In the digestion experiments in this study, CPZ in concentrations exceeding 10^{-5} Mol decreased the intracellular brake down of engulfed Candida particles, as measured by the release of metabolize ^{125}Iodine and observed morphologically. Disruption of lysosomes with release of hydrolytic enzymes could provide the basis for the cytolytic effect on macrophages leading to impaired ability of phagocytosis as demonstrated in this study. This may also be the cause of some undesirable clinical effects which have been observed in organs which concentrate CPZ such as the liver and the central nervous system (16), where toxic manifestations like jaundice and parkinsonism may be the result of lysosomal disruption leading to cell damage.

Technical assistance from Mrs. *B. H. Hansen* is gratefully acknowledged. I am also grateful to prof. *J. Lamvik* and dr. *K. E. Viken* for critical discussion and help during the course of this study and the preparation of the manuscript. This work was supported by grants from the *Norwegian Cancer Society* and from the *Norwegian Research Council for Science and the Humanities*. The author is a Research Fellow of the County of Sør-Trøndelag.

REFERENCES

1. *Boyum, A.*: Separation of leucocytes from blood and bone marrow. Scand. J. Clin. Lab. Invest. *21*: Suppl. 97, 1968.
2. *Cohn, Z. A.* & *Wiener, E.*: The particulate hydrolases of macrophages. I Comparative enzymology, isolation and properties. J. Exp Med. *118*: 991–1008, 1963.
3. *Fand, I.*: Effects of phenothiazine tranquillizers on lysosomes in cultured human cells. Arch. Int. Pharmacodyn. *183*: 247–260, 1970.
4. *Frisk-Holmberg, M.* & *Unnäs, B.*: The influence of Chlorpromazine on the uptake of biogenic amines by rat mast cells in vitro. Acta Phys. Scand. *86*: 1–11, 1972.

5. *Goldman, D.:* The major complications of treatment of psychotic states with Chlorpromazine and Reserpine and their management. Psychiatr. Res. Rep. Am. Psychiatr. Assoc. *4:* 79–88, 1956.
6. *Guth, P. S. & Spirtes, M. A.:* The phenothiazine tranquillizers, biochemical and biophysical actions. Int. Rev. Neurobiol. *7:* 231–278, 1964.
7. *Kvarstein, B. & Stormorken, H.:* Influence of Acetylsalicylic acid, Butazolidine, Colchichine, Hydrocortisone, Chlorpromazine and Imipramine on the phagocytosis of polystyrene latex particles by human leucocytes. Bioch. Pharm. *20:* 119–124, 1971.
8. *Landmark, K. & Øye, I.:* The action of Thioridazine and Promazine on biological membranes, relationship between ATP-ase inhibition and membrane stabilization. Acta Pharmacol. et Toxicol. 29: 1–8, 1971.
9. *Munro, A., Bewick, M., Mauel, L., Cameron, J. S., Ellis, F. G., Boulton-Jones, M. & Ogg, C. S.:* Clinical evalution of a rosette inhibition test in renal allotransplantation. *Br. Med. J. 3:* 271–275, 1971.
10. *Preston, F. E. & Lee, D.:* The effect of phenothiazines on human erythrocytes, an ultrastructural study. Br. J. Haematol. *20:* 563–566, 1971.
11. *Ross, L. M.:* The cell. Ciba Clinical Symposia Vol 25, No. 4, 1973, p. 28.
12. *Ruutu, T.:* Effect of phenothiazine and related compounds on phagocytosis and bacterial killing by human neutrophilic leucocytes. Ann. Med. Exp. Biol. Fenn. *50:* 24–36, 1972.
13. *Ruutu, T. & Collan, Y.:* Short-term action of Chlorpromazine on human leucocytes and erythrocytes. Ann. Med. Exp. Biol. Fenn. *50:* 42–48, 1972.
14. *Sanderson, A. R.:* Application of iso-immune cytolysis using radiolabelled target cells. Nature (Lond.) *204:* 250–253, 1964.
15. *Santos-Martinez, J., Aviles, T. A. & Laboy-Torres:* The haemolytic effect of some phenothiazine derivatives in vitro and in vivo. Arch. Int. Pharmocodyn. *196:* 83–92, 1972.
16. *Seeman, P.:* Membrane stabilization by drugs, tranquillizers, steroids and anesthetics. Int. Rev. Neurobiol. *9:* 145–221, 1966.
17. *Seeman, P. & Kwant, W. O.:* Membrane expansion of the erythrocyte by both the neutral and ionized form of Chlorpromazine. Biochem. Biophys. Acta *183:* 512–519, 1969.
18. *Spirtes, M. A. & Guth, P. S.:* Effects of Chlorpromazine on biological membranes. I. Chlorpromazine-induced changes in liver mitochondria. Biochem. Pharmacol. *12:* 37–46, 1963.
19. *Viken, K. E.:* ^{125}I-labelling of *Candida albicans* by electrolysis. Acta path. microbiol. scand. Sect. B, *82:* 219–222, 1974.
20. *Viken, K. E. & Ødegaard, A.:* Phagocytosis of heat-killed radiolabelled *Candida albicans* by human blood monocytes cultured in vitro. Acta path. microbiol. scand. Sect. B, *82:* 235–244, 1974.
21. *Ødegaard, A., Viken, K. E. & Lamvik, J.:* Structural and functional properties of blood monocytes cultured in vitro. Acta path. microbiol. scand. Sect. B, *82:* 223–234, 1974.

and ultrastructural defects in the cell membrane have been demonstrated by other workers (10, 13).

Chlorpromazine in a concentration of 10^{-6} or 10^{-5} Mol did not appear to influence the intracellular digestion of particles as observed morphologically. At 10^{-5} Mol a reduced release of ^{125}Iodine to the cell free medium was found, indicating a stabilisation of the cell membrane with reduced transfer of ^{125}Iodine to the medium. Many authors have demonstrated that CPZ within the above mentioned range of concentrations is an effective inhibitor of active and passive transport of different molecules through biological membranes (4, 8). *Landmark* & *Oye* (8) demonstrated that CPZ decreased the loss of K^+ from erythrocytes and isolated rat heart cells. They concluded that the drug effect could not be due to a direct inhibition of the active K^+ transport mechanism, but was secondary to more general alterations in the physical properties of the cell membrane. *Spirtes* & *Guth* found that CPZ could stabilize the lysosome fraction of rat liver cells with up to 50 per cent inhibition of the leakage of acid phosphatase (18).

Lysosomes which have completed their digestive function and have become filled with debris are called residual bodies. There is evidence that residual bodies may fuse with the cell membrane and extrude their contents from the cells by a process of reversed pinocytosis called exoplasmosis (11). The observed membrane alterations described in this study, possibly reflect extruded material from the cells and could be a morphological counterpart to exoplasmosis. However, until the chemical and ultrastructural characteristics of this material have been further analysed, it is impossible to conclude what the membrane alterations really reflects. The observation that Chlorpromazine could inhibit the formation of these membrane projections indicate that the mechanism is dependent upon a normal function of the cell surface membrane.

Human monocytes cultured *in vitro* improve their digestive capacities concomitant with an increase in the phase-dense granules locates in the perinuclear region of the cells (21). These granules have been shown to be lysosomes containing hydrolytic enzymes (2). Lytic concentrations of CPZ has been found to increase the permeability of lysosomes in bone marrow cells, resulting in a release of enzymes to the periphery of cell cytoplasm, as demonstrated by *Fand* (3). In the digestion experiments in this study, CPZ in concentrations exceeding 10^{-5} Mol decreased the intracellular brake down of engulfed Candida particles, as measured by the release of metabolize ^{125}Iodine and observed morphologically. Disruption of lysosomes with release of hydrolytic enzymes could provide the basis for the cytolytic effect on macrophages leading to impaired ability of phagocytosis as demonstrated in this study. This may also be the cause of some undesirable clinical effects which have been observed in organs which concentrate CPZ such as the liver and the central nervous system (16), where toxic manifestations like jaundice and parkinsonism may be the result of lysosomal disruption leading to cell damage.

Technical assistance from Mrs. *B. H. Hansen* is gratefully acknowledged. I am also grateful to prof. *J. Lamvik* and dr. *K. E. Viken* for critical discussion and help during the course of this study and the preparation of the manuscript. This work was supported by grants from the *Norwegian Cancer Society* and from the *Norwegian Research Council for Science and the Humanities*. The author is a Research Fellow of the County of Sør-Trøndelag.

REFERENCES

1. *Boyum, A.*: Separation of leucocytes from blood and bone marrow. Scand. J. Clin. Lab. Invest. *21*: Suppl. 97, 1968.
2. *Cohn, Z. A.* & *Wiener, E.*: The particulate hydrolases of macrophages. I. Comparative enzymology, isolation and properties. J. Exp Med. *118*: 991–1008, 1963.
3. *Fand, I.*: Effects of phenothhiazine tranquillizers on lysosomes in cultured human cells. Arch. Int. Pharmacodyn. *183*: 247–260, 1970.
4. *Frisk-Holmberg, M.* & *Unnås, B.*: The influence of Chlorpromazine on the uptake of biogenic amines by rat mast cells in vitro Acta Phys. Scand. *86*: 1–11, 1972.

5. *Goldman, D.:* The major complications of treatment of psychotic states with Chlorpromazine and Reserpine and their management. Psychiatr. Res. Rep Am. Psychiatr. Assoc. *4:* 79–88, 1956.
6. *Guth, P. S. & Spirtes, M. A.:* The phenothiazine tranquillizers, biochemical and biophysical actions. Int. Rev. Neurobiol. *7:* 231–278, 1964.
7. *Kvarstein, B. & Stormorken, H.:* Influence of Acetylsalicylic acid, Butazolidine, Colchichine, Hydrocortisone, Chlorpromazine and Imipramine on the phagocytosis of polystyrene latex particles by human leucocytes. Bioch. Pharm. *20:* 119–124, 1971.
8. *Landmark, K. & Øye, I.:* The action of Thioridazine and Promazine on biological membranes, relationship between ATP-ase inhibition and membrane stabilization. Acta Pharmacol. et Toxicol. *29:* 1–8, 1971.
9. *Munro, A., Bewick, M., Mauel, L., Cameron, J. S., Ellis, F. G., Boulton-Jones, M. & Ogg, C. S.:* Clinical evalution of a rosette inhibition test in renal allotransplantation *Br. Med. J. 3:* 271–275, 1971.
10. *Preston, F. E. & Lee, D.:* The effect of phenothiazines on human erythrocytes, an ultrastructural study. Br. J. Haematol. *20:* 563–566, 1971.
11. *Ross, L. M.:* The cell. Ciba Clinical Symposia Vol. 25, No. 4, 1973, p. 28.
12. *Ruutu, T.:* Effect of phenothiazine and related compounds on phagocytosis and bacterial killing by human neutrophilic leucocytes. Ann. Med. Exp. Biol. Fenn. *50:* 24–36, 1972.
13. *Ruutu, T. & Collan, Y.:* Short-term action of Chlorpromazine on human leucocytes and erythrocytes. Ann. Med. Exp. Biol. Fenn. *50:* 42–48, 1972.
14. *Sanderson, A. R.:* Application of iso-immune cytolysis using radiolabelled target cells. Nature (Lond.) *204:* 250–253, 1964.
15. *Santos-Martinez, J., Aviles, T. A. & Laboy-Torres:* The haemolytic effect of some phenothiazine derivatives in vitro and in vivo. Arch. Int. Pharmocodyn. *196:* 83–92, 1972.
16. *Seeman, P.:* Membrane stabilization by drugs, tranquillizers, steroids and anesthetics. Int. Rev. Neurobiol. *9:* 145–221, 1966.
17. *Seeman, P. & Kwant, W. O.:* Membrane expansion of the erythrocyte by both the neutral and ionized form of Chlorpromazine. Biochem. Biophys. Acta *183:* 512–519, 1969.
18. *Spirtes, M. A. & Guth, P. S.:* Effects of Chlorpromazine on biological membranes. I. Chlorpromazine-induced changes in liver mitochondria. Biochem. Pharmacol. *12:* 37–46, 1963.
19. *Viken, K. E.:* ^{125}I-labelling of *Candida albicans* by electrolysis. Acta path. microbiol. scand. Sect. B, *82:* 219–222, 1974.
20. *Viken, K. E. & Odegaard, A.:* Phagocytosis of heat-killed radiolabelled *Candida albicans* by human blood monocytes cultured in vitro. Acta path. microbiol. scand. Sect. B, *82:* 235–244, 1974.
21. *Odegaard, A., Viken, K. E. & Lamvik, J.:* Structural and functional properties of blood monocytes cultured in vitro. Acta path. microbiol. scand. Sect. B, *82:* 223–234, 1974.

Acta path. microbiol. scand. Sect. C, *83:* 74–82, 1975

THE EFFECT OF AZATHIOPRINE ON MONONUCLEAR BLOOD CELLS CULTURED *IN VITRO*

ARNE ØDEGAARD

Trondheim Central Hospital, Department of Medicine, Section for Haematology and Immunology, Trondheim, Norway

Ødegaard, A. The effect of azathioprine on mononuclear blood cells cultured in vitro. Acta path. microbiol. scand. Sect. C, *83:* 74–82, 1975.

The effect of Azathioprine (AZP), an immunosuppressive drug, on the function of blood monocytes and lymphocytes cultured *in vitro*, was studied. An attempt was made to correlate the effect of the drug on various functions of mononuclear phagocytes with the effect on the rosette forming ability of lymphocytes. The experiments indicate that AZP in concentrations below the level of cytotoxicity suppressed the rosette forming ability of lymphocytes as well as the digestive capacity of macrophages.

Key words: Mononuclear blood cells; effect of azathioprine.

A. Ødegaard, Department of Medicine, Trondheim Central Hospital, 7000 Trondheim, Norway.

Received 13.viii.74 Accepted 22.viii.74

There is evidence that AZP reduces the immune response at concentrations which are too small to be clearly antiproliferative (11). It has been shown that AZP primarily depresses the primary immune response (2), and that prolonged tolerance in animals only could be obtained when the drug was given during the induction period of the immune response. There are also indications that AZP affects the inflammatory reaction.

Macrophages appear to be of importance in the afferent and efferent limb of the immunological reaction. The first step in antigen recognition may consist in processing of antigen by macrophages. Labelling experiments have shown that the tissue macrophages originate from the blood monocytes (5). Monocytes from human blood undergo a similar differentiation when cultured *in vitro* (3, 14). After being processed by macrophages antigen or antigenic information appear to cause lymphocyte alterations, which eventually give the different expressions of immunity. The aim of the present work was to study the effect of AZP on cells of importance in the first steps of immune reactions, namely macrophages and T-lymphocytes. A test system with mononuclear blood cells cultured *in vitro* was used with standardized techniques for the registration of lymphocyte and monocyte functions.

MATERIALS AND METHODS

The general methods for culturing of human monocytes and for registration of drug effect on different functions of mononuclear blood cells have been reported in detail in a previous report (13, 14). Registration of cellular adhesiveness, preparation of rosette-forming lymphocytes and registra-

TABLE 1. *Effect of Azathioprine on the Adhesiveness of Blood Mononuclear Cells after 90 Minutes' Exposure to the Drug*

Culture series no.	Cell numbers added $\times 10^6$	Concentrations of AZP, mg/ml 0.5	0.125	0.0156	0 0078	0.0039	Control without AZP
73	7.5	1.4	2.0	–	–	–	1.5
74	8.6	1.8	1.2	1.4	1.2	0.9	0.9
75	9.2	3.7	4.3	3.3	3.2	3.2	3.2
76	5.8	0.9	1.1	1.5	1.2	1.3	1.2
77	8 3	2.8	2.7	2.9	3.3	3.4	2.6
78	7.5	2.1	2.7	2.2	3.2	2.4	1.2

Numbers of adhesive cells per culture dish $\times 10^6$, estimated as the difference between the numbers of cells added to the culture dishes and the numbers of cells removed after 90 minutes' incubation.
– not tested.

TABLE 2. *Effects of Azathioprine on Survival of Blood Mononuclear Phagocytes Cultured* in Vitro

Culture series no.	Concentrations of AZP, mg/ml 0.5	0.125	0.0156	0 0078	0 0039	Control without drug
73	0	24.4	22.1	–	–	11.3
74	0	40.3	20.5	15.5	14.1	13.5
75	0	16.4	13.6	11.7	7.6	7.1
76	0	8.6	14.0	9.4	8.8	7.8
77	0	16 8	19.3	–	14 0	17.1
78	0	6.5	5.1	4.5	4.5	3.3

The cells were exposed to the drug for 90 minutes at culture start and the numbers of adhesive phagocytes counted after 4 days' culturing. The values given are the means of the cell numbers $\times 10^4$ in triplicate culture dishes.
– not tested.

tion of survival of mononuclear adhesive cells after exposure to the drug were performed as described in test model I (13).

Monocytes cultured on coverslips were tested for phagocytosis after 8 days' culturing. The effect of Azathioprine on engulfment and on digestion of radiolabelled heat-killed Candida particles was studied in separate experiments and registrated according to methods described previously as test model II (13). Azathioprine sodium (Imuran, kindly supplied by Burroughs Wellcome) was dissolved in and diluted with sterile distilled water.

In the engulfment experiments, the cells were incubated with a constant number of Candida particles and AZP in varying concentrations. The cells were harvested after a period of phagocytosis of 10 minutes. The ratio between the number of Candida particles added and the number of adhesive cells was usually about 20:1

In the digestion experiments, a constant number of Candida was added to 8 days old coverslips cultures After a further incubation for 10 minutes the coverslips were washed 3 times and placed in new Petri dishes containing fresh culture medium. AZP in varying concentrations was added to the culture medium one hour later. The cultures were harvested after 24 hours' incubation. The radioactivity in the supernatant and in the sediment following high speed centrifugation was measured separately. The radioactivity on the coverslips was registered as a measure of the amount of Candida left.

RESULTS

Cellular Adhesiveness

Two different methods have been used to investigate the effect of Azathioprine on cellular adhesiveness. The data given in Table 1

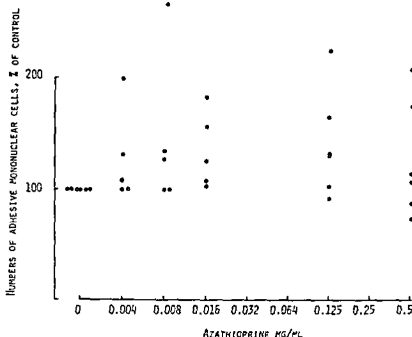


Fig. 1. The numbers of adhesive mononuclear blood cells in plastic Petri dishes after an incubation period of 90 minutes with different concentrations of AZP in the culture medium. The numbers of adhesive cells were estimated as the difference between the numbers of cells added to the culture dishes and the numbers of cells removed after 90 minutes' incubation. The values given are the means of the cell numbers in triplicate culture dishes, and are expressed as percentages of the control values without AZP added.

TABLE 3. *Effect of Azathioprine on the Rosette-formation of Human Lymphocytes After a Pre-incubation Period of 90 Minutes with Different Concentrations of Drug Added to the Culture Medium*

Culture series no.	Concentrations of AZP, mg/ml					Control without drug
	0.5	0.125	0.0156	0.0078	0.0039	
73	0	1.5	–	–	–	10.8
74	0	10.4	15.8	12.5	17.0	21.4
75	0	4.0	11.4	17.0	10.8	17.4
76	0	9.5	30.2	27.8	35.6	38.0
77	0	4.5	13.8	13.9	18.4	19.0
78	0	12.5	25.4	31.6	20.6	27.0
79	0	11.3	25.5	–	22.6	25.8

The numbers are given as percentages of the total lymphocyte count.
– not tested

are based upon an objective registration by counting cells in an electronic particle counter; the data given in Table 2 are based upon direct cell counting in a phase contrast microscope.

Azathioprine in a final concentration between 0.004 mg/ml and 0.5 mg/ml did not reduce the adhesiveness of mononuclear blood cells to plastic surface at culture start (Fig. 1, Table 1). The cell numbers given in Table 2, Fig. 2, indicate that concentrations of the drug up to 0.125 mg/ml did not cause

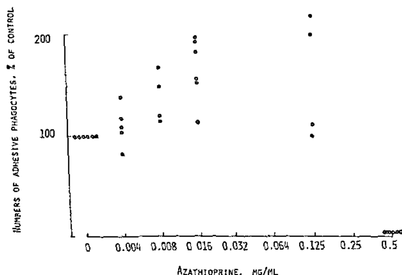
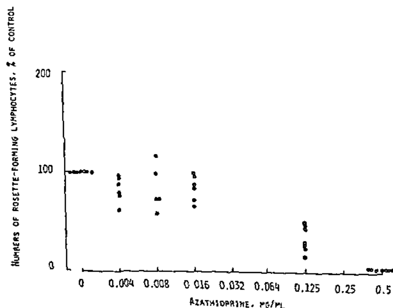


Fig. 2. The numbers of adhesive mononuclear phagocytes after an incubation period of 90 minutes with different concentrations of AZP in the culture medium. The numbers of adhesive cells were counted after 4 days' culturing. The values given are the means of the cell numbers in triplicate culture dishes, and are expressed as percentages of the control samples without AZP added.



ig 3. The numbers of rosette forming lymphocytes after a pre-incubation period of 90 minutes with lifferent concentrations of AZP in the culture medium. The values given are expressed as percentages of the control samples without AZP added.

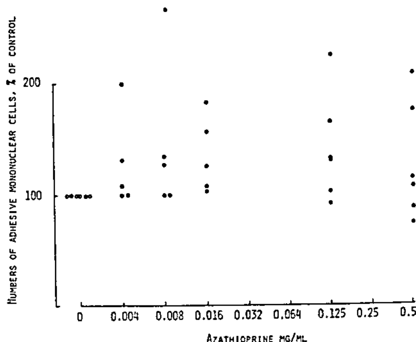


Fig. 1. The numbers of adhesive mononuclear blood cells in plastic Petri dishes after an incubation period of 90 minutes with different concentrations of AZP in the culture medium. The numbers of adhesive cells were estimated as the difference between the numbers of cells added to the culture dishes and the numbers of cells removed after 90 minutes' incubation. The values given are the means of the cell numbers in triplicate culture dishes, and are expressed as percentages of the control values without AZP added.

TABLE 3. *Effect of Azathioprine on the Rosette-formation of Human Lymphocytes After a Pre-incubation Period of 90 Minutes with Different Concentrations of Drug Added to the Culture Medium*

Culture series no.	Concentrations of AZP, mg/ml					Control without drug
	0.5	0.125	0.0156	0.0078	0.0039	
73	0	1.5	–	–	–	10.8
74	0	10.4	15.8	12.5	17.0	21.4
75	0	4.0	11.4	17.0	10.8	17.4
76	0	9.5	30.2	27.8	35.6	38.0
77	0	4.5	13.8	13.9	18.4	19.0
78	0	12 5	25.4	31.6	20.6	27.0
79	0	11.3	25.5	–	22.6	25.8

The numbers are given as percentages of the total lymphocyte count.
– not tested

are based upon an objective registration by counting cells in an electronic particle counter; the data given in Table 2 are based upon direct cell counting in a phase contrast microscope.

Azathioprine in a final concentration between 0 004 mg/ml and 0.5 mg/ml did not reduce the adhesiveness of mononuclear blood cells to plastic surface at culture start (Fig. 1, Table 1). The cell numbers given in Table 2, Fig. 2, indicate that concentrations of the drug up to 0.125 mg/ml did not cause

TABLE 5. *Effect of Different Concentrations of Azathioprine on the Digestion of Engulfed Radiolabelled Heat-killed Candida in 8 Days Old Blood Mononuclear Phagocytes Cultured in Vitro on Glass Coverslips*

Concentrations of AZP, mg/ml	Radioactivity: coverslips ct/min × 10²	coverslips %*	sediment ct/min × 10²	sediment %*	cell free medium ct/min × 10²	cell free medium %*	Total per culture dish ct/min × 10²
	4 3	5	80	54	58	41	144
0.5	3 3	4	81	54	62	42	148
	7 7	11	57	43	60	46	131
	13 21	26	30	23	64	51	128
0.125	27 28	31	38	22	83	47	176
	19 21	29	21	14	80	57	141
	17 27	29	9	6	98	65	151
0.0156	17 16	23	9	7	100	70	142
	27 19	34	8	6	81	60	135
	19 19	30	6	5	80	65	124
0.0078	22 16	28	8	6	88	66	134
	15 23	16	10	7	96	67	144
	14 18	23	7	4	103	73	142
0.0039	8 28	35	6	6	63	59	105
	16 19	27	4	5	83	68	122
	17 15	38	8	8	46	54	86
Control without drug	6 21	22	10	8	87	70	124
	11 21	26	5	4	86	70	123

The figures listed are the results from one typical digestion experiment performed in triplicate culture dishes.

* per cent of total radioactivity per culture dish.

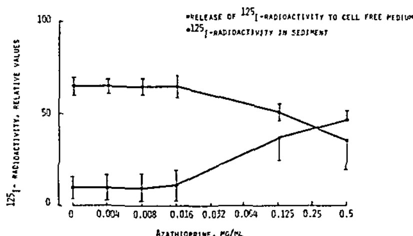


Fig. 5. Effect of AZP on digestion of ^{125}I-labelled heat-killed Candida particles in blood mononuclear phagocytes cultured for 8 days on coverslips. The values are the means of the results from 6 experiments performed in triplicate culture dishes and registrated 23 hours following the engulfment phase. Standard deviations are marked as bars.

the cells on two coverslips in each culture dish. The total radioactivity in each culture dish reflects the numbers of engulfed particles in the phagocytes before addition of the drug. The ability of the cells to digest Candida and transfer metabolized ^{125}Iodine was quantified as the percentage of the added radioactivity found in the cell free medium at harvesting. As shown previously (12), radiolabelled Candida particles will passively release approximately 12 per cent radioactivity when incubated for 1 to 3 days. This passive release was not corrected for in the values listed in Fig 5 and Table 5.

Azathioprine in a final concentration between 0.125 and 0.5 mg/ml decreased the release of ^{125}Iodine to cell free medium, indicating a reduced intra-cellular digestion of the engulfed particles. Concomitant with this reduction, an increase in the radioactivity in the sediment was found. Microscopic examination of this sediment showed detached cells containing undigested Candida particles.

DISCUSSION

Azathioprine is widely used as an immunosuppressive agent in man. In some clinical disorders it appears to be superior to other cytostatic drugs, being less toxic and more effective in preventing rejection of kidney transplants and in the treatment of autoimmune diseases (4). As an antimetabolite, AZP interferes with the normal metabolic pathway involving nucleic acids, leading to an inhibition of RNA and DNA synthesis (7).

Its superiority as an immunosuppressant is not completely understood although it has been shown to suppress both cellular and humoral immunological reactions (11). AZP has been shown to inhibit the rosette formation of lymphocytes with sheep red blood cells and this property has been used to assay the concentration of the drug in the serum of patients (1). AZP is also known to inhibit inflammation and it has been reported that patients receiving the drug after organ transplantations often suffer from serious pulmonary infections (6). It has been proposed that AZP in concentrations used therapeutically in man primarily affects the inflammatory reaction (10).

In this study an attempt was made to correlate the effect of AZP on various functions of mononuclear phagocytes with the effect on the rosette forming ability of

lymphocytes. AZP in a concentration of 0.125 mg/ml reduced the number of rosette forming lymphocytes by more than 50 per cent. This concentration of the drug did not appear to influence the adhesiveness or survival of the mononuclear phagocytes incubated with AZP for the same period of time.

Monocytes cultured *in vitro* on glass surfaces differentiate into macrophages (3, 14). As shown in this study, the engulfment of radio-labelled Candida particles in mononuclear phagocytes was not affected by AZP. On the other hand, the drug caused an impaired ability of the cells to digest the engulfed particles. This observation is in accordance with the findings by *Lockard et al.*[6] namely that alveolar macrophages from rats which had received cytostatic drugs showed impaired digestive capacities towards bacteria whereas the engulfment phase remained normal (8). The main source of alveolar macrophages has been shown to be the circulating blood monocyte (5). Infections associated with immunosuppressive therapy may thus be linked to a deficiency in the ability of macrophages to destruct ingested organism. Suppression of cell proliferation may be of less importance. In the treatment of auto-immune diseases, *Borel & Schwartz* (2) found that bone marrow depression was not necessary for immunosuppression. They demonstrated that AZP only depressed the primary immune response.

There is evidence that the macrophages cooperate with the lymphocyte in the initiation of the immune response. On the other hand, these cells are involved in the maintenance of chronic inflammatory reaction. Therefore, it may be difficult to distinguish the effect of AZP on immunity from its effect on inflammatory reaction. There is, however, clinical and experimental evidence that the drug both affects the inflammatory reaction and acts on different sites in the immune response involving lymphocytes and macrophages. The present findings also give evidence for a multi-potential mode of action. AZP below the level of cytotoxicity suppressed the rosette forming ability of lymphocytes as well as the digestive capacity of macrophages.

[6] Acta path. microbiol. scand. Sect. C 83, 1

Technical assistance from Mrs. *B. H. Hansen* is gratefully acknowledged. This work was supported by grants from the *Norwegian Cancer Society* and from the *Norwegian Research Council for Science and the Humanities*. The author is a Research Fellow of the County of Sør-Trøndelag.

REFERENCES

1. *Bach, J. F., Dardenne, M. & Fournier, C.:* In vitro evaluation of immunosuppressive drugs. Nature (Lond.) *222:* 998–999, 1969.
2. *Borel, Y. & Schwartz, R.:* Inhibition of immediate and delayed hypersensitivity in the rabbit by 6-mercaptopurine. J. Immunol. *92:* 754–761, 1964.
3. *Cohn, Z. A. & Wiener, E.:* The particulate hydrolases of macrophages. I. Comparative enzymology, isolation and properties. J. Exp. Med. *118:* 991–1008, 1963.
4. *Elion, G. B.:* Significance of Azathioprine metabolites. Soc. Roy. Soc. Med. *65:* 257–259, 1972.
5. *van Furth, R. & Cohn, Z. A.:* The origin and kinetics of mononuclear phagocytes. J. Exp. Med. *128:* 415–435, 1968.
6. *Hill, R. B., Rowlands, D. T. & Rifkind, D.:* Infectious pulmonary diseases in patients receiving immunosuppressive therapy for organ transplantation. N. Eng. J. Med. *271:* 1021–1027, 1964.
7. *Hitchings, G. H. & Elion, G. B.:* The role of antimetabolites in immunosuppression and transplantation. Accounts of Chemical Research *2:* 202–209, 1969.
8. *Lockard, V. G., Sharbaugh, R. J., Arhelger, R. B. & Grogan, J. B.:* Ultrastructural alterations in phagocytic functions of alveolar macrophages after Cyclophosphamide administration. RES-Journal of Reticuloendothelial Society *9:* 97–107, 1971.
9. *Munro, A., Bewick, M., Manuel, L., Cameron, J. S., Ellis, F. G., Boulton-Jones, M. & Ogg, C. S.:* Clinical evaluation of a rosette inhibition test in renal allotransplantation. Br. Med. J *3:* 271–275, 1971.
10. *Roitt, I.:* Essential immunology. 1 ed. Blackwell Scientific Publications, London 1971, p. 165
11. *Swansen, M. A. & Schwartz, R.:* Immunosuppressive therapy. N. Eng. J. Med. *277.* 163–170, 1967.
12. *Viken, K. E. & Odegaard, A..* Phagocytosis of heat-killed radiolabelled Candida albicans

by human blood monocytes cultured in vitro. Acta path. microbiol. scand. Sect. B, *82:* 235–244, 1974.

13. *Odegaard, A.:* Functional alterations induced by Chlorpromazine in mononuclear blood cells cultured in vitro. Acta path. microbiol. scand. Sect. C, *83:* 59–73, 1975.

14. *Odegaard, A., Viken, K. E. & Lamvik, J.:* Structural and functional properties of blood monocytes cultured in vitro. Acta path. microbiol. scand. Sect. B, *82:* 223–234, 1974.

Acta path. microbiol scand. Sect. C, *83:* 83–94, 1975

APPEARANCE OF ACID PHOSPHATASE STAINING IN SENSITIZED LYMPHOID CELLS DURING THEIR LYSIS OF ALLOGENEIC FIBROBLASTS

Poul Brix Poulsen, Flemming Güttler and Peer Nobert Jorgensen

Institute of Medical Microbiology, University of Copenhagen, Denmark

Poulsen, P. Brix, Güttler, F. & Jørgensen, P. Nobert. Appearance of acid phosphatase staining in sensitized lymphoid cells during their lysis of allogeneic fibroblasts. Acta path. microbiol. scand. Sect. C, 83: 83–94, 1975.

Immune spleen cells or activated thymus cells were incubated with appropriate ^{51}Cr-labelled target fibroblasts. The interaction of lymphoid cells and target cells was stopped by fixation in formalin vapour. Target cell lysis was evaluated by the release of ^{51}Cr and the cultures were stained for acid phosphatase activity by the simultaneous capture technique, using naphthol AS-BI phosphoric acid and Fast red-violet LB salt. The sensitized lymphoid cells aggregated around cultured donor-type fibroblasts within a 4-hour incubation period and staining for acid phosphatase activity appeared in the lymphocytes. The reaction was immunologically specific, as staining was observed only in the immune spleen cells or activated thymocytes incubated with target cells possessing the allo-antigens to which the cell donor was sensitized. Specific adsorption on fibroblast monolayers syngeneic, but not allogeneic, to the H-2 allo-antigens against which the thymus cells were activated, reduced both the specific release of ^{51}Cr from labelled target cells and the number of thymus cells staining for acid phosphatase activity. Acid phosphatase staining and cytolytic activity were reduced in lymphocyte-target cell cultures incubated in the presence of prednisolone or heparin. A significant correlation was found between target cell lysis and number of acid phosphatase-positive spleen cells in relation to time of interaction and lymphocyte: target cell ratio.

Key words. Lymphoid cells; acid phosphatase staining; allogeneic fibroblasts; lysis.

F. Güttler, Institute of Medical Microbiology, University of Copenhagen, 22 Juliane Maries Vej, DK-2100 Copenhagen Ø, Denmark.

Received 19.vii.74   Accepted 28.x.74

Spleen cells from allo-immunized mice are cytotoxic to cultured foetal mice fibroblasts bearing the appropriate allo-antigens (*Jørgensen et al.* 1974). Target cell lysis mediated in this system is immunologically specific and requires an intimate association between thymus-processed lymphocytes and the target cell (*Cerottini & Brunner* 1974). This type of cytolytic reactions are believed to be *in vitro* correlates to corresponding effector activities of lymphoid cells *in vivo* (*Perlman & Holm* 1969; *Cerottini & Brunner* 1974). Despite the accumulation of a great number

Part of this material was presented at The V Annual Meeting of The Scandinavian Society for Immunology, Copenhagen 1974.

of data relative to the requiremenets for cytolosis in this system, the mechanism by which cytolysis is effected remains obscure (*Henny & Bubbers* 1973; *Martz & Benacerraf* 1973; *Cerottini & Brunner* 1974). It is established that the lymphocytes must be viable (*Rosenau* 1963) and capable of protein synthesis (*Mauel et al.* 1970).

In a recent review, *Weissmann & Dukor* (1971) state that histochemical studies of transformed lymphoid cells engaged in the destruction of target tissue have shown that the invading lymphocytes are rich in a variety of primary and secondary lysosomes. Stabilizers of lysosome membranes, especially glucocorticoid hormones, have been shown to inhibit lymphocyte-mediated injury of target cells (*Mauel et al.* 1970; *Cohen et al.* 1971). Based on a more extensive study employing a variety of stabilizers of lysosome membranes and inhibitors of lysosomal enzyme, *Brondz et al.* (1971, 1973) suggest that activation of lysosomal enzymes might be an important step in the series of events leading to target cell destruction. In the present study, histochemical methods were used in order to examine the hypothesis that lysosomal enzymes might have a role in cell-mediated target cell injury.

MATERIAL AND METHODS

Animals Mice of inbred strains C3H/HeJ and DBA/2J were obtained from the Jackson Laboratory, Bar Harbor, Maine, U.S.A. F_1 hybrids of C3H/HeJ × DBA/2J were bred at the Institute of Medical Microbiology, Copenhagen.

Chemicals Cytochalasin B was obtained from Aldrich Europe, Janssen Pharmaceutica N.V, Belgium, and dissolved in dimethylsulphoxide at a concentration of 10 mg/ml. Heparin was purchased as sodium-mucoitinpolysulphate from NOVO Industries Ltd, Denmark. Prednisolone was obtained as prednisolone sodium succinate from Hoechst, Germany *Naphthol AS-BI phosphoric* acid (N-2125, no 420-4160), Fast red-violet LB salt (F-1625, no 420-2740), and actinomycin D were purchased from the Sigma Chemical Company, St. Louis, Mo, U S.A.

Tumours The following tumours used for alloimmunization (or iso-immunization as controls) were obtained from the Jackson Laboratory: H-2^k: 6C3HED, C HBA H-2^b BW 10232, C 1498. The P-815-X2 mastocytoma of the H-2^d origin was kindly donated by Dr. K. T. Brunner and Dr. J.-C. Cerottini, Lausanne, Switzerland.

Immunization and preparation of spleen cell suspensions. Recipient mice were injected intraperitoneally with 5×10^6 allogeneic (or syngeneic) tumour cells. The spleens were removed aseptically 9 to 10 days later and homogenized by hand in an all-glass Ten-Broek grinder. The cell suspension obtained was allowed to sediment for 45 min at 4° C. The supernatant fluid was centrifuged at $500 \times g$ for 10 min and the sediment was washed four times in Parker's medium without serum added and finally resuspended in Parker's medium with serum. The lymphocyte suspension was adjusted to 10^7 cells per millilitre.

Preparation and activation of thymus cells. The thymuses of parental mice were removed aseptically and homogenized by one stroke by hand in a Ten-Broek grinder. The cell suspension was washed in Parker's medium supplemented with 10 per cent (v/v) inactivated foetal calf serum (*Paul* 1965) and adjusted to contain 10^8 viable thymus cells per ml. One ml of this solution was injected into the tailvein of lethally irradiated (800 r) F_1 hybrids The spleens of the F_1 hybrids reconstituted with parental thymus cells were removed six days later and homogenized in a Ten-Broek grinder. The cell suspension obtained was allowed to sediment for 45 min at 4° C and the supernatant fluid was washed four times, as described above

Target cells. Primary cultures of mice foetal skin fibroblasts were obtained as described by *Paul* (1965) and subcultured twice before use as target cells. Parker's medium (*Paul* 1965) supplemented with 10 per cent (v/v) foetal calf serum and antibiotics, was used as the basal culture medium throughout this study. The cells were maintained in Kolle flasks. For histochemical examination, 10^5 cells were cutured overnight on coverslips in Leighton tubes at 37° C. Spleen cells from immunized mice were added in amounts of 25, 50, and 100 times 10^5 in 1 ml of culture medium. The time of interaction was evaluated from this point

Labelling of target cells. For labelling, the cells were scraped off and separated mechanically. The technique described by *Cerottini & Brunner* (1974) was followed by incubating 5×10^6 cells in 1 ml of medium containing 50 μCi ^{51}Cr as Na_2CrO_4 (sp. act. >200 mCi/mg) at 37° C for ½ h. After five washes, the cells were resuspended in medium at a concentration of 10^5 cells per millilitre.

Labelling of activated thymus cells. The cell suspension of activated thymus cells obtained as described above was incubated in a concentration of 5×10^6 cells in 1 ml of Parker's medium containing 150 mCi ^{51}Cr as Na_2CrO_4 (sp. act >200 mCi/mg, The Radiochemical Centre, Amersham, England) at 37° C for 45 min. After five

washes, the labelled thymus cells were resuspended in medium at a concentration of 3×10^6 cells per 0.3 ml of medium.

Irradiation of activated thymus cells. 10^7 activated thymus cells were resuspended in 10 ml of culture medium and pipetted into an 85 mm plastic petri dish. The dish was placed in a Siemens Stabilipan therapy machine operated at 220 kV, 15 mA, with an HVL of 1.5 mm of copper. The rate of irradiation was 46 R/min.

Adsorption on fibroblast monolayers. DBA/2 foetal fibroblasts or L-cells of C3H origin were grown to confluency in Falcon flasks, 75 cm^2. Activated thymus cells were seeded on the monolayers at a number of 15–20 $\times 10^6$ cells per Falcon flask. The flasks were gently agitated every half hour and, after incubation for three hours, the non-adsorbed cells were poured off, counted for number of viable mononuclear cells, tested for cytotoxicity, and stained for acid phosphatase activity.

Quantitation of ^{51}Cr release. Volumes of 0.6 ml of reaction mixtures containing 3×10^4 labelled target cells and 25, 50, and 100 times 3×10^4 lymphoid cells were pipetted off into test tubes set up in roller drums (20 rotations per hour) and incubated for 4 h at 37° C. Five cultures were incubated for each mixture. At the termination of the reaction, the tubes were centrifuged at 700 $\times$ *g* for 10 min and 0.4 ml of the supernatant was added to the scintillation fluid after which the radioactivity was counted in a Beckman LS-233 liquid scintillation counter. The efficiency was approximately 40 per cent. The spontaneous release of ^{51}Cr was measured in cultures incubated with 0.6 ml of medium in place of lymphocyte suspension. For each target preparation, total ^{51}Cr release was measured by adding 3×10^5 labelled target cells to the scintillation fluid containing the non-ionic detergent BBS-3 (Beckman).

Histochemistry. Acid phosphatase activity was demonstrated using the simultaneous capture reaction (*Barka & Anderson* 1962). The interaction of lymphoid cells and target cells was terminated by fixation in formalin vapour as follows: the coverslips with target cells and lymphoid cells were carefully removed from the Leighton tubes used for culturing and placed for 5 min on glass cotton over a 40 per cent formaldehyde solution in a 12 $\times$ 65 mm test tube at 37° C. Then the coverslips were incubated for 10 hours at 37° C in the naphthol AS-BI phosphoric acid-Fast red-violet LB substrate according to *Barka & Anderson* (1962). Naphthol AS-BI phosphoric acid was dissolved in 0.2 M-sodium acetate buffer, pH 5.0, at a final concentration of 0.5 mg/ml and mixed with Fast red-violet LB salt dissolved in 9 per cent HCl. (MERCK), the final concentration being 2 mg/ml. After incubation, the coverslips were placed on a slide, sealed, and immediately photographed under bright-field microscopy at magnifications of 32 or 128 times (Fig. 1). In order to quantitate the acid phosphatase staining, the clusters of aggregated lymphoid cells around target cells were scored by the proportion of lymphoid cells staining for acid phosphatase activity. The clusters of lymphoid cells within four fields of 3 mm^2 were ranked according to percentage of acid phosphatase-positive cells. The scores obtained by independent readers rarely varied more than 25 per cent.

Cytolytic assay in the presence of inhibitors. Activated thymus cells and target fibroblasts were incubated in the presence of the inhibitor. Aliquots of a stock solution of the inhibitor were diluted in culture medium to give the following concentrations of the inhibitor per ml of culture medium: Prednisolone 5 μg/ml, heparin 100 i.e./ml, cytohalasin B 10 μg/ml, actinomycin D 10 μg/ml. Incubations were carried out at 37° C in Leighton tubes for subsequent assay of acid phosphatase activity and in test tubes set up in rollar drums for quantitation of specific release of ^{51}Cr-labelled target cells.

RESULTS

Comments on the histochemical method used. Preliminary experiments revealed that fixation in conventional aqueous fixatives such as the formol-calcium solution recommended by *Barka & Anderson* (1962) detached the lymphoid cells expected to adhere to target cells. We also recognized that, following incubation of target cells with sensitized lymphoid cells for six hours or longer, the target cells began to detach from the coverslips surrounded by a cluster of adhering lymphoid cells, thus eluding our analysis. Similar phenomena were observed during application of conventional techniques for dehydration and mounting. Thus, after fixation in formalin vapour and staining for acid phosphatases, the coverslip was placed on a slide, sealed, and immediately photographed. Target fibroblasts fixed and stained four hours after medium change without addition of lymphocytes revealed the red colour of the azo dye localized to known sites of enzyme activity, i.e. granules concentrated in the perinuclear area (Fig. 2). Obvious artifacts such as nuclear staining or diffuse staining of the cytoplasma were not observed (Fig. 2).

Acid phosphatase staining of lymphocyte-target cell cultures. The immune spleen cells aggregated around cultured donor-type fibroblasts within a four-hour incubation period (Figs. 3 and 4). Apart from the perinuclear acid phosphatase-positive granules in target fibroblasts, a number of the aggregated spleen cells showed a marked staining for acid phosphatase activity (Figs. 3 and 4). Very few of the immune spleen cells showed acid phosphatase staining when incubated on foetal fibroblasts not sharing major H-2 allo-antigens with the tumour allograft used for sensitization. The perinuclear staining of the target fibroblasts served as a positive control of the histochemical reaction (Fig. 5).

Quantitation of the acid phosphatase stain-

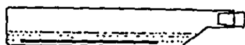










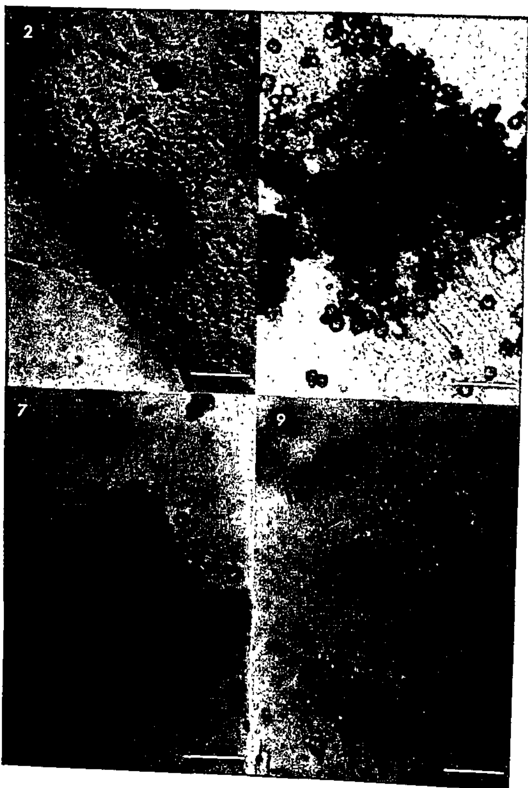
Fig. 1. Schematic representation of the experimental design to examine acid phosphatase staining in sensitized lymphoid cells during lysis of allogeneic fibroblasts cultured on coverslips in Leighton tubes [illegible] Phosphatase activity was demonstrated by [illegible] simultaneous capture technique, using naphthol AS-BI phosphoric acid and Fast red-violet LB salt. Target cell lysis was evaluated by release of ^{51}Cr from the labelled fibroblasts. a) Target cells and lymphoid cells incubated on coverslip for 4 hours at 37° C; b) coverslip placed in 40 per cent formalin vapour on glass wool for 5 min at 37° C; c) coverslip placed in incubating medium for acid phosphatase staining for 10 hours at 37° C. Thereafter the coverslip was placed under microscope and photographed.

Fig. 2. Cultured C3H fibroblast fixed and stained four hours after medium change without addition of spleen cells. Naphthol AS-BI phosphoric acid—Fast red-violet LB salt. Magnification 128 times. —— 25 μ.

Fig. 3. Acid phosphatase staining of a culture of C3H foetal fibroblasts incubated for four hours with immune spleen cells at a ratio of 50 spleen cells per target cell. Naphthol AS-BI phosphoric acid—Fast red-violet LB salt. Magnification 128 times. —— 25 μ.

Fig. 7. Acid phosphatase staining of a culture of C3H cells incubated for four hours with DBA/2 thymus cells activated against the C3H allotype. Prior to incubation with target cells the thymus cells were adsorbed on a DBA/2 monolayer, as indicated in Fig. 6. Naphthol AS-BI phosphoric acid —Fast red-violet LB salt. Magnification 128 times. —— 25 μ.

Fig. 9. Thymus cells activated against the C3H allotype and non-adsorbed on a C3H monolayer were incubated with C3H target fibroblasts and stained for acid phosphatase activity, using naphthol AS-BI phosphoric acid—Fast red-violet LB salt. Magnification 128 times. —— 25 μ.



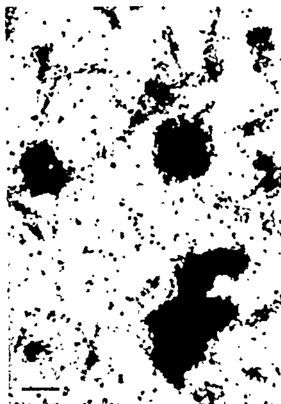


Fig. 4. Spleen cell of C57BL/6 mice sensitized by a C3H tumour allograft and incubated for four hours on C3H foetal fibroblasts in a ratio of 50 spleen cells per target cell. The naphthol AS-BI phosphoric acid—Fast red-violet LB salt revealed red coloured clusters of lymphoid cells adhering to the target fibroblasts. Magnification 32 times. —— 25 μ.



Fig. 5. Sensitized spleen cells obtained as in Fig. 4 but incubated on DBA/2 foetal fibroblasts not sharing major histocompatibility allo-antigens with the C3H donor strain. Naphthol AS-BI phosphoric acid—Fast red-violet LB salt. Magnification 32 times. —— 25 μ.

TABLE 1. *Clusters of Immune Spleen Cells around Cultured Donor-type Fibroblasts Scored by the Proportion of Leucocytes Staining for Acid Phosphatase Activity*

Lymphocyte/Target cell ratio	Percentage of acid phosphatase-positive leucocytes* >75	75–50	50–25	25–5	<5
10/1	1	0	0	5	2
20/1	2	3	2	2	0
40/1	6	8	5	5	2
80/1	10	6	4	3	3

* The values given are the average number of clusters of four fields of 3 mm². The cultures were stained for acid phosphatase activity after a 4-hour incubation period.

ing. The red coloured clusters of lymphoid cells adhering to target cells (Fig. 4) were scored by the proportion of lymphoid cells staining for acid phosphatase activity. If ranked according to the percentage of lymphocytes staining for acid phosphatase activity, the percentage of acid phosphatase-positive leucocytes per cluster of aggregated

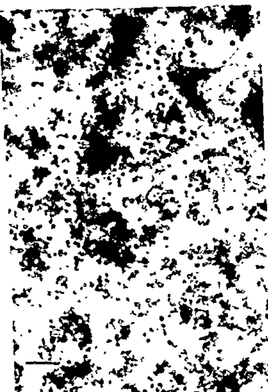


Fig. 6. Acid phosphatase staining of DBA/2 thymus cells activated against the C3H allotype and non-adsorbed on DBA/2 fibroblasts. The activated thymus cells were before incubation with C3H target cells seeded on syngeneic DBA/2 fibroblasts and incubated for three hours. The non-adsorbed cells were used in a ratio of 50 thymus cells per C3H target cell. Naphthol AS-BI phosphoric acid —Fast red-violet LB salt. Magnification 32 times. —— 25 μ.

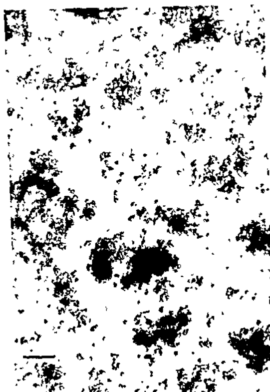


Fig. 8. DBA/2 thymus cells activated against the C3H allotype were adsorbed on a C3H monolayer for three hours. The non-adsorbed cells were incubated with C3H target cells for four hours. After fixation the cultures were stained for acid phosphatase activity, using the naphthol AS-BI phosphoric acid—Fast red-violet LB salt technique. The proportion of non-adsorbed thymus cells per target cell was 50 to 1. Magnification 32 times. —— 25 μ.

spleen cells tended to increase by increasing the proportion of immune spleen cells to target cells (Table 1).

Kinetics of the acid phosphatase staining. By varying the lymphocyte-to-target-cell ratio it was found that the number of spleen cell clusters with 50 per cent or more acid phosphatase-positive leucocytes correlated with the number of target cells destroyed as evaluated by the amount of ^{51}Cr released from labelled target cells (Table 2). A positive correlation between specific release of ^{51}Cr from the labelled target cells and number of acid phosphatase-positive spleen cells in relation to time of interaction between the immune spleen cells and appropriate target cells was formed (Table 3).

Acid phosphatase staining of activated thymus cells. Lethally irradiated [C3H × DBA/2]F_1 hybrids were reconstituted by DBA/2 thymus cells. Six days later the DBA/2 cells activated against the recipient C3H allo-antigens were harvested from the spleens. Half of the cell suspension of activated cells was seeded on syngeneic DBA/2 fibroblasts and the other half of the activated cells was seeded on C3H fibroblasts. After incubation for three hours, the non-adhering thymocytes were harvested from the supernatant fluid and incubated with C3H target

TABLE 2. *Number of Spleen Cell Clusters with 50 Per Cent or more Acid Phosphatase-positive Leucocytes around ^{51}Cr-labelled Target Cells in Relation to the Percentage Specific ^{51}Cr Release*

Lymphocyte/Target cell ratio	No. of spleen cell clusters*		Percentage ^{51}Cr release†
	immune	non-immune	
10/1	1	0	–3
20/1	5	0	6
40/1	14	0	13

* The clusters of spleen cells formed within 4 hours were stained and counted as in Table 1.
† Percentage specific ^{51}Cr release (immune lymphocytes—non-immune lymphocytes) over the net total release (total ^{51}Cr incorporated—non-immune lymphocytes) according to the formula [(I-N)/(T-N)] × 100.

TABLE 3. *The Relationship between Time of Incubation with Target Cells and Appearance of Clusters of Immune Spleen Cells with 50 Per cent or more Acid Phosphatase-positive Leucocytes*

Time of incubation (hours)	No. of clusters per 3 mm²*	Percentage ^{51}Cr release†
1/2	3	. .
1	8	0.1
2	7	2
3	15	3
4	14	13

* Measured as in Table 1.
† Calculated as in Table 2.

cells. The percentage of non-adsorbed cells recovered was comparable in the two situations (Table 4). Thymus cells activated against C3H allo-antigens and adsorbed on syngeneic DBA/2 fibroblasts followed by incubation with C3H target fibroblasts revealed a marked staining for acid phosphatase activity (Figs. 6 and 7 and Table 4). If thymus cells activated against the alloantigens of the C3H strain were adsorbed on C3H monolayers and the non-adsorbed cells were incubated with C3H target cells, a significant decrease in the number of thymocytes staining for acid phosphatase activity was found (Figs. 8 and 9 and Table 4).

The influence of inhibitors. In order to test further the possible relationship between acid phosphatase staining of sensitized lymphocytes and their lysis of target cells, we examined the influence of some drugs known to inhibit cell-mediated cytotoxicity. The percentage decrease in specific release of ^{51}Cr of cultures incubated with the inhibitor compared to cultures incubated without inhibitor was used as a measure of the inhibitory effect of the drug. There was good correlation between the percentage of reduction in release of ^{51}Cr and decrease in number of sensitized thymocytes staining for acid phosphatase ac-

TABLE 4 *Staining for Acid Phosphatase Activity Related to Cytotoxicity of Activated Thymus Cells after Specific Adsorption on Fibroblast Monolayers*

Type of thymocytes	Monolayers used for adsorption	No. of clusters by non-adsorbed cells*	Percentage ^{51}Cr release by non-adsorbed cells†	Percentage non-adsorbed cells recovered
DBA/2→(C3H × DBA/2)	C3H	8	55	62
DBA/2→(C3H × DBA/2)	DBA/2	47	90	52

* Average number of thymus cell clusters per 3 mm² with 50 per cent or more acid phosphatase-positive lymphocytes after a 4-hour incubation period on C3H fibroblasts.
† Release from ^{51}Cr-labelled C3H fibroblasts after an 18-hour incubation period calculated according to the formula [(experimental—spontaneous)/(total—spontaneous)] × 100.

TABLE 5. *Effect of Inhibitors of Cell Mediated Cytotoxicity on Acid Phosphatase Staining of Activated Thymus Cells and Target Cell Lysis*

Type of treatment*	Acid phosphatase staining†	Per cent reduction of cytotoxicity§
None (control)	+ + +	
Actinomycin D (10 μg/ml)	+	76.8
None (control)	+ + +	
Prednisolone (5 μg/ml)	—	79 2
Heparin (100 i.e./ml)	+	84.2
None (control)	+ + +	
Cytochalasin B (10 μg/ml)	+ +	49.8
Prednisolone (5 μg/ml)	+ +	29.0
X-irradiation (800 r.)$	+ + +	0 0

* Activated thymus cells were pre-incubated with the inhibitor for half an hour at 37° C and the suspension added to target cell cultures without washing.

† The reactions were scored on a scale of—to 4+, reflecting the percentage of aggregated leucocytes displaying acid phosphatase staining after a 4-hour incubation period, namely: 0, 25, 50, 75, or 100 per cent.

§ Reduction of cytotoxicity is expressed as: 100 minus per cent specific cytotoxicity of activated thymus cells in cultures containing the inhibitor over the specific cytotoxicity in cultures with no inhibitor added. The percentage specific activity was calculated as indicated in Table 4.

$ X-irradiation of thymus cells before incubation with target cells.

tivity (Table 5). Irradiation by 800 r of activated thymus cells prior to incubation with target cells reduced neither the cytotoxic effect nor the staining for acid phosphatase activity.

^{51}Cr-labelling of activated thymus cells. In order to examine whether sensitized lymphoid cells might be injured during their interaction with target cells, activated thymus cells were labelled with ^{51}Cr and incubated with target cells either syngeneic or allogeneic (controls) to the H-2 allotype against which the thymus cells were sensitized. Unlabelled sensitized thymus cells incubated with the appropriate ^{51}Cr-labelled target cells served as controls of the specific cytotoxicity of the thymus cells employed. The specific release of ^{51}Cr from ^{51}Cr-labelled sensitized thymus cells incubated with the appropriate unlabelled target was 1 per cent, compared to 2 per cent if the labelled thymus cells were incubated with unrelated target cells.

DISCUSSION

The complexities of the homograft response *in vivo*, i.e. vascular incursions and necrotic zones, are absent in the *in vitro* tissue culture system which isolates from the homograft response the interaction of lymphoid cells and target cells. The present observations demonstrate the appearance of staining for acid phosphatase activity in immune spleen cells or activated thymus cells incubated with the appropriate target fibroblasts *in vitro*. The reaction was immunologically specific and a significant correlation between target cell lysis and number of acid phosphatase positive lymphocytes in relation to time of interaction and lymphocyte: target cell ratio was found. Adsorption on fibroblast monolayers syngeneic, but not allogeneic, to the H-2 allo-antigens against which thymus cells were activated, reduced both the specific release of ^{51}Cr from labelled target cells and the number of thymus cells staining for acid phosphatase activity.

Specific adsorption of sensitized lymphoid cells on monolayers of cells bearing the immunizing allo-antigens was first demonstrated by *Brondz* (1964). *Golstein et al.* (1971 and 1972), and *Berke & Levey* (1972) demonstrated the existence of receptor-bearing immune lymphocytes involved in target cell lysis by *specific adsorption and subsequent elution* of these cells. Based on these observations and on the inhibition experiments referred to below, the process of lymphocyte-mediated target cell lysis may be divided into at least three stages: recognition of antigen by receptors of the surface of the lymphocyte, the killing phase in which the adsorbed sensitized lymphocytes initiate the sequence of events leading to target cell injury, and finally, the subsequent cytolytic processes within the dying cell (*Cohen et al.* 1971; *Golstein & Blomgren* 1973; *Henney & Bubbers* 1973; *Martz & Benacerraf* 1973).

Lymphoid cells of human peripheral blood are cytotoxic to target cells if cultured in the presence of phytohemagglutinin (*Perlmann & Holm* 1969). The cytotoxic lymphoid cells often adhere to the target cells by the site of the lymphocyte which contains the centrosome Golgi area (*Biberfeld* 1971). *Biberfeld* (1971) showed that the uropod by which lymphocytes are seen to attach to target cells contains lysosomes. Histochemical examinations have demonstrated increased acid phosphatase activity in lymphoid cells stimulated by PHA (*Hirschhorn et al.* 1965 and 1967). The PHA-induced increase in acid phosphatase activity is observed during the late phase of lymphocyte stimulation, i.e. it reaches a maximum after 2–3 days of incubation (*Cohnen et al.* 1973). PHA-induced cytolysis can be detected after a few hours and is completed within 24 hours after incubation of PHA-stimulated lymphoid cells with ^{51}Cr-labelled target cells (*Perlmann & Holm* 1969). In the present study, smears of sensitized spleen cells or thymus cells did not stain for acid phosphatase activity (unpublished observation). However, acid phosphatase-positive lymphoid cells could be observed after incubation with appropriate target cells for half an hour and were easily detected after incubation with target cells for two hours. These observations suggest that activation of acid phosphatases of lymphoid cells mediating cytotoxicity may occur during the early phase of lymphocyte-target cell interaction

Lymphoid cells sensitized *in vivo* can be reactivated *in vitro* if the donor allotype used for sensitization acts as stimulator cell in a mixed lymphocyte culture reaction (*Cerottini & Brunner* 1974 *Heron* 1974). Cultured foetal fibroblasts can equally well sensitize lymphoid cells *in vitro* (*Cohen et al.* 1971). The present finding of activation of acid phosphatases in sensitized lymphoid cells seeded on the appropriate target fibroblasts may reflect an intracellular process occurring during *in vitro* reactivation.

Rosenau & Moon (1962) have demonstrated that hydrocortisone prevents cytolysis without inhibiting antigenspecific clustering of lymphocytes around target cells. *Brondz et al.* (1973) showed that prednisolone and heparin inhibit cell mediated cytotoxicity without inhibiting the initial specific adsorption of immune lymphocytes to target cells. In addition, *Cohen et al.* (1971) found that the cytolytic capacity per cell of prednisolone treated lymphocytes, after the hormone was removed, was increased as compared with lymphocytes sensitized in the absence of added hormone. The present observations of reduced acid phosphatase staining in lymphocyte-target cell cultures incubated in the presence of prednisolone or heparin may indicate that acid phosphatases of sensitized lymphoid cells mediating target cell lysis are activated distal to the recognition phase. Our observations that irradiation of activated thymus cells prior to incubation with target cells reduced neither the cytotoxic effect nor the staining for acid phosphatases, further support this suggestion and are in harmony with findings obtained by *Stobo et al.* (1973). *Stobo et al.* (1973) showed that sublethal irradiation (350 r) completely ablated the develepment of cytolytically active spleen cells from mice immunized prior to irradiation.

Studies by *Henney* (1973), *Plaut et al.* (1973), and *Martz & Benacerraf* (1973) suggest that the killing phase of lymphocyte-target cell interaction occurs within 15–30 min of interaction and that some enzymatic reaction is involved in the terminal phase of lymphocyte mediated killing (*Martz & Benacerraf* 1973). The findings by *Henney et al.* (1972), and *Strom et al.* (1972) suggest that cAMP and cGMP occupy a central modulatory role in the expression of cytolytic activity by lymphocytes, and further that one of the steps during cell mediated killing might be considered "secretory". Furthermore, an activated esterase may have an essential role in the events leading to target cell lysis (*Ferluga et al.* 1972). The possibility that lysosomal acid hydrolases in lymphocytes are released in proximity to plasma membranes and have a role in the killing phase of lymphocyte mediated cytotoxicity (*Cohnen et al.* 1973),

may be supported by the present study (cf. Fig. 3).

A regular microscopic observation has been that lymphoid cells from donors sensitized to target cell antigens aggregate to the target cell (cf. *Perlmann* & *Holm* 1969). Aggregation usually precedes morphological changes and death of the target cell (*Rosenau* & *Moon* 1961). Our findings are in agreement with these observations. In agreement with the present data are also the findings by *Wilson* (1965) suggesting that lymphoid cells are not killed in the course of the cytotoxic reaction. From data supplied by *Cerottini* & *Brunner* (1974), and *Golstein* (1974) it may be concluded that thymus-processed lymphocytes trigger lysis of target cells in the *in vitro* model of the allograft reaction. It has been extrapolated that 1–2 per cent of lymphoid cells from lymph nodes or the thoracic duct of allografted rats were immunologically active, i.e. specifically sensitized effector cells (*Wilson* 1965). The number of acid phosphatase-positive lymphoid cells observed in the present study far exceeded this estimate of number of allo-aggressive cells, even when activated thymus cells were used. However, the relative role in *in vitro* cytotoxicity of bystander lymphoid cells is not known.

Target cell killing may be brought about by local release of "mediators" liberated from specifically sensitized effector lymphocytes in contact with the target cell (cf. *Perlmann* & *Holm* 1969; *Holm et al.* 1973). Such "mediators" secreted during lymphocyte-mediated target cell lysis might be responsible for secondary aggregation of bystander lymphoid cells (*Asherson* & *Ferluga* 1973) and for activation of acid phosphatases in the lymphoid cells sticking to the target cell.

Six iso-enzymes with acid phosphatase activity have been demonstrated in PHA-stimulated lymphoid cells (*Kaluinsky* & *Lindquist* 1973). Recently, *Bowers* (1972) has detected that thoracic duct lymphocytes contain 10 acid hydrolases known to be lysosomal in other tissues. Further investigations are required in order to provide insight into the question whether activation of lysosomal acid hydrolases may be a coincident phenomenon, the consequence or probably the cause of target cell lysis.

The authors are indebted to Dr. *Bent Rubin* and Dr. *Ebbe Dickmeiss* for valuable advice and help during the adsorption experiments and experiments involving inhibitors of cell-mediated cytotoxicity, and to Mrs. *Kirsten Florentz* for her excellent technical assistance. The authors express their thanks to *The Danish Medical Research Council* for financial support (Grants Nos 512-817, 512-1164 and 512-4045). *Poul Brix Poulsen* was the recipient of a scholarship from The University of Copenhagen. *Peer Nobert Jorgensen* is the recipient of a Research Fellowship from the University of Copenhagen.

REFERENCES

Asherson, G. L. & *Ferluga, J.:* Contact sensitivity in the mouse. X. Non-specific cytotoxicity of T blasts in the draining lymph nodes. Immunology *25:* 471–483, 1973.

Barka, T. & *Anderson, P. J.:* Histochemical methods for acid phosphatase using hexazonium pararosanilin as coupler. J. Histochem. Cytochem *10:* 741–753, 1962.

Berke, G. & *Levey, R. H.:* Cellular immunoadsorbents in transplantation immunity. Specific in vitro deletion and recovery of mouse lymphoid cells sensitized against allogeneic tumors. J. exp. Med. *135:* 972–981, 1972.

Biberfeld, P.: Cytotoxic interaction of phytohemagglutinin-stimulated blood lymphocytes with monolayer cells: A study by light and electron microscopy. Cell Immunol. *2:* 54–72, 1971.

Bowers, W. E.: Lysosomes in rat thoracic duct lymphocytes. J. exp Med. *136:* 1394–1403, 1972.

Brondz, B. D.: Interaction of immune lymphocytes in vitro with normal and neoplastic tissue cells. Folia biol. (Prague) *10:* 164–173, 1964.

Brondz, B. D., Snegirõva, A. E., Rassulin, I. A. & *Shamborant, O. G.:* Effect of some enzymes, polysaccharides, and lysosome-active drugs on interaction of immune lymphocytes with allogeneic target cells. In: Amos, B. (Ed.): Progress in Immunology. Academic Press, New York 1971, p. 447–460.

Brondz, B. D., Snegirõva, A. E., Rassulin, I. A. & *Shamborant, O. G.:* Modification of in vitro immune lymphocyte-target cell interaction by some biologically active drugs. Immunochemistry *10:* 175–189, 1973.

Cerottini, J.-C. & *Brunner, K. T.:* Cell-mediated cytotoxicity, allograft rejection and tumor immunity. Adv. Immunol. *18:* 67–132, 1974.

Lymphoid cells of human peripheral blood are cytotoxic to target cells if cultured in the presence of phytohemagglutinin (*Perlmann* & *Holm* 1969). The cytotoxic lymphoid cells often adhere to the target cells by the site of the lymphocyte which contains the centrosome Golgi area (*Biberfeld* 1971). *Biberfeld* (1971) showed that the uropod by which lymphocytes are seen to attach to target cells contains lysosomes. Histochemical examinations have demonstrated increased acid phosphatase activity in lymphoid cells stimulated by PHA (*Hirschhorn et al.* 1965 and 1967). The PHA-induced increase in acid phosphatase activity is observed during the late phase of lymphocyte stimulation, i.e. it reaches a maximum after 2–3 days of incubation (*Cohnen et al.* 1973). PHA-induced cytolysis can be detected after a few hours and is completed within 24 hours after incubation of PHA-stimulated lymphoid cells with ^{51}Cr-labelled target cells (*Perlmann* & *Holm* 1969). In the present study, smears of sensitized spleen cells or thymus cells did not stain for acid phosphatase activity (unpublished observation). However, acid phosphatase-positive lymphoid cells could be observed after incubation with appropriate target cells for half an hour and were easily detected after incubation with target cells for two hours. These observations suggest that activation of acid phosphatases of lymphoid cells mediating cytotoxicity may occur during the early phase of lymphocyte-target cell interaction

Lymphoid cells sensitized *in vivo* can be reactivated *in vitro* if the donor allotype used for sensitization acts as stimulator cell in a mixed lymphocyte culture reaction (*Cerottini* & *Brunner* 1974, *Heron* 1974). Cultured foetal fibroblasts can equally well sensitize lymphoid cells *in vitro* (*Cohen et al.* 1971). The present finding of activation of acid phosphatases in sensitized lymphoid cells seeded on the appropriate target fibroblasts may reflect an intracellular process occurring during *in vitro* reactivation

Rosenau & *Moon* (1962) have demonstrated that hydrocortisone prevents cytolysis without inhibiting antigenspecific clustering of lymphocytes around target cells. *Brondz et al.* (1973) showed that prednisolone and heparin inhibit cell mediated cytotoxicity without inhibiting the initial specific adsorption of immune lymphocytes to target cells. In addition, *Cohen et al.* (1971) found that the cytolytic capacity per cell of prednisolone treated lymphocytes, after the hormone was removed, was increased as compared with lymphocytes sensitized in the absence of added hormone. The present observations of reduced acid phosphatase staining in lymphocyte-target cell cultures incubated in the presence of prednisolone or heparin may indicate that acid phosphatases of sensitized lymphoid cells mediating target cell lysis are activated distal to the recognition phase. Our observations that irradiation of activated thymus cells prior to incubation with target cells reduced neither the cytotoxic effect nor the staining for acid phosphatases, further support this suggestion and are in harmony with findings obtained by *Stobo et al.* (1973). *Stobo et al.* (1973) showed that sublethal irradiation (350 r) completely ablated the develepment of cytolytically active spleen cells from mice immunized prior to irradiation.

Studies by *Henney* (1973), *Plaut et al.* (1973), and *Martz* & *Benacerraf* (1973) suggest that the killing phase of lymphocyte-target cell interaction occurs within 15–30 min of interaction and that some enzymatic reaction is involved in the terminal phase of lymphocyte mediated killing (*Martz* & *Benacerraf* 1973). The findings by *Henney et al.* (1972), and *Strom et al.* (1972) suggest that cAMP and cGMP occupy a central modulatory role in the expression of cytolytic activity by lymphocytes, and further that one of the steps during cell mediated killing might be considered "secretory". Furthermore, an activated esterase may have an essential role in the events leading to target cell lysis (*Ferluga et al.* 1972). The possibility that lysosomal acid hydrolases in lymphocytes are released in proximity to plasma membranes and have a role in the killing phase of lymphocyte mediated cytotoxicity (*Cohnen et al.* 1973),

Acta path. microbiol. scand. Sect. C, *83:* 95–96, 1975

BRIEF REPORT

T-LYMPHOCYTE DEPLETED MICE WITH A NORMAL B-LYMPHOCYTE RESPONSE SHOW NORMAL AMYLOID DEVELOPMENT AFTER CASEINATION

P. Ebbesen[1], *M. Bergh*[1], *E. Leuchars*[2] *and M. Doenhoff*[2]

[1] Institute of Medical Microbiology, 22 Juliane Maries Vej, DK-2100 Copenhagen Ø, Denmark
[2] Chester Beatty Research Institute, Fulham Rd., London SW 3, England

Ebbesen, P., Bergh, M., Leuchars, E. & Doenhoff, M. T-lymphocyte depleted mice with normal B-lymphocyte response show normal amyloid development after caseination. Acta path. microbiol. scand. Sect. C, *83:* 95–96, 1975.

Adult thymectomized, irradiated, bone marrow reconstituted CBA/Lac mice had a much reduced pool of PHA reactive (T)-lymphocytes and a normal pool of bacterial lipopolysaccharide reactive (B)-lymphocytes. After caseination the spleens of such mice showed the same degree of amyloid development as with caseinated, but otherwise untreated mice. The result is consistent with an involvement of B- but not of T-spleen lymphocytes in amyloidogenesis.

Key words: Amyloid development; B-lymphocyte response; T-lymphocyte depletion.

P. Ebbesen, Institute of Medical Microbiology, 22 Juliane Maries Vej, DK-2100 Copenhagen Ø, Denmark.

ceived 2.xii.74 Accepted 19.xii.74

Adult thymectomy and depletion of the circulat- ; T-lymphocyte pool by thymectomy plus irra- ıtion do not influence spleen amyloid develop- ɛnt in caseinated CBA/Lac mice (*Nielsen et al.* 73). This contrasts with other observations ıich indicate a role of thymus and T-lymphocytes amyloidogenesis (*Ranlov* 1966; *Hardt & Claës- n* 1972). As spleen reticulo-endothelial and most ɛly also other as yet unidentified spleen cells are strumental in spleen amyloid formation (*Teilum* 356; *Christensen & Hjort* 1960; *Zucker-Franklin . Franklin* 1970), we have tested for a correlation ɛtween the size of the T- and B-spleen lymphocyte ɔol of thymectomized irradiated CBA/Lac mice ıd the spleen amyloid development in such mice fter caseination.

Eight-week-old CBA/Lac mice were thymecto- ıized, irradiated with 850 r one week later, and ıven different doses of syngeneic bone marrow BM) cells intravenously (*Davies et al* 1966).)ne week later, casein was administered in a man- er previously shown to induce grade 3 amyloid in ormal mice within 30 days (*Christensen & Hjort* 960). Thirty days after BM grafting, spleen cells of non-caseinated control mice were tested for *in vitro* reactivity to phytohaemagglutinin (PHA) and bacterial lipopolysaccharide (LPS), these having been shown to be relatively specific mitogens for T and B lymphocytes, respectively (*Doenhoff et al.* 1974). In brief, spleen cells from the CBA/Lac mice were cultured with an equal number of syngeneic CBA/H-T6T6 mouse spleen cells in the presence of either PHA or LPS and, three days later, the *proportion* of cells of each karyotype in metaphase was determined. A normal response from each cell type results in the finding that equal numbers of each cell type are in mitosis (*Doenhoff* 1971).

Infiltrates of lymphoid cells were found in liver sinuses of the mice grafted 2 × 10⁷ BM cells, and not in other animals. With respect to the response to caseination, all groups reacted with development of the same amount of spleen amyloid. It can be seen in Table 1 that the responsiveness of *untreated* CBA/Lac mouse spleen cells to PHA and PLS for both mitogens is slightly less than that of normal CBA/H-T6T6 mouse spleen cells. However, the PHA responsiveness of *thymectomized, irradiated, bone marrow reconstituted* CBA/Lac mice is much

TABLE 1. *Spleen Amyloid Development and* in vitro *Reactivity of Spleen Cells to PHA and LPS of Normal or Adult Thymectomized, Lethally Irradiated CBA/Lac Mice Grafted with Different Numbers of Syngeneic Bone Marrow (BM) Cells*

Pretreatment		No. of *mice*	Spleen amyloid		Spleen leucocyte *in vitro* reactivity* (mean of five)	
			Range	Mean	PHA	LPS
None						
	Casein	18	3.5	(2–4)		
	NaCl (control)	10	0		43 %	41 %
Irradiation + BM cells						
10^6	Casein	15	3.5	(3–5)		
	NaCl	5	0			
5×10^6	Casein	15	3.5	(3–5)		
	NaCl	5	0			
	Casein	15	3.5	(3–5)		
	NaCl	5	0			
Thymectomy + *irradiation* + BM cells						
10^6	Casein	15	4.2	(3–5)		
	NaCl	5	0		6 %	43 %
5×10^6	Casein	15	3.5	(2–5)		
	NaCl	5	0		4 %	37 %
2×10^7	Casein	15	4.2	(3–5)		
	NaCl	5	0		6 %	43 %

* A normal response is about 50 per cent.

reduced relative to CBA/H-T6T6 mice, though the responsiveness of PLS is relatively intact.

Since spleen amyloid production proceeded normally in mice presenting a much reduced pool of T-lymphocytes and a normal pool of B-lymphocytes in the spleen, we consider B-lymphocytes (*Ebbesen et al* 1969), and not T-lymphocytes, likely participants in the amyloidogenesis.

A grant from *Rigsforeningen til Gigtens Bekæmpelse* is gratefully acknowledged.

References. Christensen, H. E. & Hjort, G. H.: Acta path. microbiol scand *48*: 1–12, 1960.—*Davies, A. J. S., Leuchars, E., Wallis, V. & Koller, P. C.*: Transplantation *4*: 438–451, 1966.—*Doenhoff, M. J.*: Clin. exp. Immunol. *8*. 603–615, 1971. —*Doenhoff, M. J., Jarossy, G., Greaves, M. F. & Gomer, K. J.*: Clin. exp. Immunol. *17*: 475–490, 1974.—*Ebbesen, P., Schiødt, T. & Christensen, H. E.*: Cancer Res. *29*: 1851–1858, 1969.—*Hardt, F. & Claësson, M. H.*: Immunology *22*: 677–683, 1972.—*Nielsen, G., Leuchars, E., Doenhoff, M. & Ebbesen, P.*: Acta path microbiol scand. Sect. B, *81*: 242–244, 1973.—*Ranløv, P. J.*: Acta path. microbiol. scand. *67*: 42–54, 1966.—*Teilum, G.*: Amer. J. Path. *32*: 945–959, 1956.—*Zucker-Franklin, D. & Franklin, E. C.*: Amer. J Path. *59*: 23–41, 1970.

Acta path. microbiol. scand. Sect. C, 83: 97–105, 1975

CAPSULAR SWELLING AND PASSIVE HAEMAGGLUTINATION INDUCED BY MONOCLONAL IgM REACTING WITH ACID POLYSACCHARIDES OF *KLEBSIELLA*

MORTEN HARBOE, JOHANNA DEVERILL and JORUNN ERIKSEN

University of Oslo, Institute for Experimental Medical Research, Ullevål Hospital, Oslo 1 and Kaptein W. Wilhelmsen og Frues Bakteriologiske Institutt, Rikshospitalet, Oslo 1, Norway

Harboe, M., Deverill, J. & Eriksen, J. Capsular swelling and passive haemagglutination induced by monoclonal IgM reacting with acid polysaccharides of *Klebsiella*. Acta path. microbiol. scand. Sect. C, 83: 97–105, 1975.

(1) Three selected macroglobulinaemia sera, Rø, Th, and We gave highly specific precipitin reactions in gel against type 12, type 13 and type 35 acid polysaccharides of *Klebsiella*. The same specificity was observed in more sensitive passive haemagglutination tests. The activity was confined to the monoclonal IgM present in the sera, and all of the monoclonal IgM in each serum had the characteristic activity. (2) The monoclonal IgM proteins induced typical capsular swelling reactions of similar specificity with *Klebsiella* bacteria. (3) In both precipitation, passive haemagglutination and capsular swelling reactions, the IgM Rø reacted both with type 12 and type 13 polysaccharides. Absorption experiments in situ in crossed immunoelectrophoresis indicated that the same macroglobulin molecules reacted both with type 12 and type 13 polysaccharides. This cross-reaction evident in monoclonal IgM has not been seen previously in immune antibodies against *Klebsiella* type 12 and 13.

Key words: Capsular swelling; passive haemagglutination; *Klebsiella*; monoclonal IgM.

M. Harboe, Institute for Experimental Medical Research, Ullevål Hospital, Oslo 1, Norway.

Received 28.viii.74 Accepted 14.x.74

It is well established that human monoclonal IgM proteins may interact with ligands of very different origin and physicochemical character. They react with different antigens on the red cell surface, behaving as typical cold agglutinins (7, 12) with Forssman-containing liposomes (17), and with various haptens, notably substituted benzenes like DNP and TNP (1, 8, 21). They also react with proteins, as IgG (18, 19) and albumin (13, 15), with denatured DNA (21), and with various polysaccharides, e.g. acid type specific polysaccharides from *Klebsiella* (9, 10, 11). *Metzger* (19) has defined a set of criteria which must be fulfilled to accept the reaction between a monoclonal immunoglobulin and its ligand as a model for, or an example of, an antigen-antibody reaction. These criteria have been met by monoclonal IgM proteins reacting with all of the ligands mentioned above (1, 10, 11, 13, 15, 19, 20, 21).

Among these reactions, we have particularly studied the interaction between monoclonal IgM and acid polysaccharides from

Klebsiella where very strong precipitin reactions with a striking specificity can be observed.

We have previously studied 116 sera containing a monoclonal IgM for interactions with various types of ligands (*Harboe et al.*, unpublished observations), and among these several proteins have been identified which precipitate type specific acid polysaccharides from *Klebsiella*. In the present work, 3 monoclonal IgM proteins were selected which precipitated Klebsiella type specific polysaccharides in double diffusion tests with a very clear specificity. Our purpose was to study whether agglutination of red blood cells coated with polysaccharides showed the same specificity as in the gel diffusion tests, and to examine if these monoclonal IgM proteins would induce specific capsular swelling reactions. The capsular swelling reaction is a classic technique in bacterial typing. The antisera used are typical hyperimmune sera and very little information is available on the ability of IgM antibodies to induce capsular swelling.

MATERIALS AND METHODS

Sera. One hundred and sixteen sera containing a monoclonal IgM were tested for reaction with a series of different ligands including 17 type specific acid polysaccharides from *Klebsiella* by double diffusion tests in agarose From these, sera Th, Rq. and We were selected since they gave very strong precipitin reactions with some of the 17 *Klebsiella* type specific polysaccharides. The high specificity of these reactions is illustrated by IgM We which precipitated polysaccharide 13 but none of the other poysaccharides. These sera all contained a typical monoclonal IgM protein of high concentration (above 30 mg/ml).

Macroglobulin preparations A macroglobulin containing fraction was isolated from serum We by gel filtration on Sephadex G 200. Two ml of serum were chromatographed on a 5 × 95 cm column, using an elution rate of about 20 ml/h and 0.02 M Tris-HCl buffer of pH 8 0 containing 0.32 M NaCl and 0.015 M NaN_3 Fractions corresponding to the void volume peak were pooled, concentrated, and subjected to zone electrophoresis using agarose as supporting medium. The fractions from the centre of the macroglobulin peak were tested for activity by capsular swelling reactions and used for preparation af Fabμ and Fcμ fragments by splitting with pepsin and trypsin respectively (13).

Macroglobulin containing fractions were also obtained by density gradient ultracentrifugation of the macroglobulinaemia sera as previously described (14).

Anti-κ, anti-λ, and anti-μ antisera were obtained from Dakopatts Copenhagen, Denmark.

Bacterial Antigens and Related Immunological Techniques

Bacteria. Klebsiella types 12, 13, and 35, originally obtained from The State Serum Institute, Copenhagen, were kept freeze dried.

Polysaccharides. The capsular, acid type specific polysaccharides were isolated from *Klebsiella* bacteria by cold water extraction, and the crude material was purified by repeated precipitation with cetyl pyridinium chloride until no further change in optical rotation could be observed (4). Polysaccharides obtained in this way were used as antigens.

Immune sera. Hyperimmune sera from rabbits were used as controls for the capsular swelling reaction. These sera were obtained by intravenous immunization of rabbits with whole bacteria, as described in the following paper (6). Test bleedings from the ear were examined 5–6 days after the last immunization, and heart puncture was performed the following day if the serum was satisfactory. The control sera gave specific capsular swelling reactions with titers of 1:32 or higher against the homologous bacteria.

Passive haemagglutination test. Haemagglutination tests were made using sheep red cells coated with acid polysaccharides by the chromium chloride techniques as described previously (5).

Capsular swelling reaction. The reaction was carried out as described before (16) by mixing one drop of a six-hour broth culture of *Klebsiella* with one drop of serum or serum dilution on a slide. The preparation was covered with a cover slip and examined microscopically using the oil-immersion lens. The highest serum dilution giving distinct capsular swelling was considered as the end point.

Immunological Methods

Double diffusion tests in gel. These were performed in 0.5 per cent agarose gel on 5 × 5 cm glass plates Except when otherwise indicated, the gel contained phosphate-buffered saline (PBS: 0.01 M phosphate buffer, pH 7.4, in 0.14 M NaCl). The diameter of the wells was 3 mm, and the centre to centre distance 7 mm.

Crossed immunoelectrophoresis with intermediate gel. This was performed by a technique modified from *Axelsen* (2). The plates were 8 × 8 cm, and

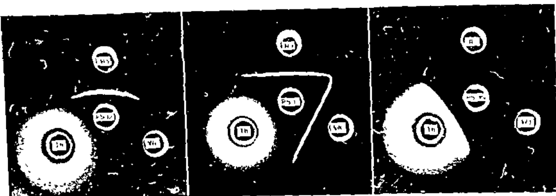


Fig. 1. Precipitin reactions between *Klebsiella* type specific polysaccharides and macroglobulinaemia sera by double diffusion tests in agarose.

the gel arrangement appears from Fig 2. Previous experiments had shown that precipitation of PS12 and PS13 by IgM Ro was critically dependent upon the buffer in the gel. Both polysaccharides were strongly precipitated by gel diffusion tests in agarose containing PBS, whereas there was no precipitation between IgM Ro and PS12 in gels containing barbiturate buffer of pH 8.6, ionic strength 0.05. The explanation of this observation is not clear, but additional experiments showed that precipitation occurred if a barbiturate/Tris buffer of pH 8.6 was used for preparation of the gel. For crossed immunoelectrophoresis 1 per cent agarose gel (agarose batch No. 115, Litex, Glostrup, Denmark) was therefore made in Tris/barbiturate buffer of the following composition: 5-5-diethyl barbituric acid 112.1 g, Tris (Sigma 7-9) 221.5 g, calcium lactate 2.7 g, sodium azide 3 2 g, dissolved in distilled water to a total volume of 5 l, and diluted 1:5 in distilled water before use.

Macroglobulinaemia serum diluted 1:8 was applied in the circular well, and the first dimension electrophoresis was run for 1¼ hours at 8 V/cm until the albumin had moved 3 cm. The intermediate gel (gel No. 2 of Fig. 2) was 1 cm wide; 0 2 ml of a polysaccharide solution containing 5 mg/ml in Tris/barbiturate buffer was mixed with 1 3 ml of 1 per cent agarose (Litex, batch No. 115). The upper gel and the lower intermediate gel (No 3 of Fig 2) were 2 5 and 4 cm wide respectively. These were also made with agarose with slight electro endosmotic flow (Litex, batch No. 115) and contained 0.1 ml anti-μ antiserum (Dakopatts, batch No. 044) per ml agarose gel. The lowest gel (No 4 of Fig. 2) was 1.5 cm wide; it was made from agarose with negligible electro endosmotic flow (Litex, batch No. 122) and contained the same concentration of anti-μ The second dimension electrophoresis was performed at 4 V/cm for 20 hours, temperature of cooling water 15° C. The plates were washed, dried, and stained with Coomassie Brilliant Blue R-250 as described by *Axelsen* (2).

This gel arrangement and the selection of different batches of agarose with varying degrees of electro endosmotic flow was based on pilot experiments attempting to establish conditions where this monoclonal macroglobulin would move towards the cathode, still providing conditions where the antibody incorporated into the gel would be kept sufficiently stable to permit precipitation to occur.

For crossed imunoelectrophoresis of Fabμ and Fcμ fragments of IgM We a slightly different arrangement was used. The intermediate gel with polysaccharide was placed at the cathodic side of the first dimension gel for experiments with the Fabμ fragment and on the anodic side in tests with the Fcμ fragment as described below.

EXPERIMENTS AND RESULTS

The precipitin reactions in gel between IgM Ro, Th, and We and the Klebsiella type specific acid polysaccharides PS12, PS13 and PS35 are illustrated in Fig. 1. The precipitin lines are very strong, and the reactions appear to be highly specific since each IgM precipitates one or two, but not all three polysaccharides. The IgM Th does not penetrate into agar gel and forms a dense precipitate along the margin of the application well. Monoclonal macroglobulins which react with agar gel in this way usually penetrate into agarose gels. Serum Th forms, however, a circular diffuse precipitate around the well in agarose gel as seen in the Figure. The precipitin line with PS35 appears as an asymmetry of the circular diffuse precipitate, but

the margin is entirely sharp and the appearance of the precipitate distinctly different in the region opposite the well containing the polysaccharide.

TABLE 1. *Agglutination of Red Cells Coated with* Klebsiella *Type Specific Polysaccharides by Macroglobulinaemia Sera*

Serum	Polysaccharide used for coating		
	Type 12	Type 13	Type 35
Ro	64,000*	32,000	–§
Th	–	–	64,000
We	–	32,000	–

* Reciprocal of highest serum dilution giving distinct agglutination.
§ Negative at dilution 1:100 and higher.

Table 1 shows the results of agglutination tests with macroglobulinaemia sera against red cells coated with *Klebsiella* type specific polysaccharides. The agglutination pattern shows a specificity identical to that observed in the precipitin tests illustrated in Fig. 1. The titres were high, 32,000 and 64,000, whereas a negative reaction means no trace of agglutination by serum diluted 1:100 or more. It was concluded from this comparison of gel precipitation and passive haemagglutination tests that monoclonal IgM proteins which react with *Klebsiella* polysaccharides can be detected in passive haemagglutination tests using chromium chloride treated red cells that have absorbed type specific polysaccharides, and that this passive haemagglutination test is characterized by marked sensitivity and retained specificity.

Demonstration of Anti-polysaccharide Activity in the Monoclonal IgM

The precipitin reactions illustrated in Fig. 1 and the passive haemagglutination tests described in Table 1 were performed with whole macroglobulinaemia sera. Experiments were then made to demonstrate that the reaction with the polysaccharides was due to the monoclonal IgM present in the sera.

Fig. 2 shows the findings by crossed immunoelectrophoresis of serum Ro in agarose gel containing anti-μ with intermediate gels containing *Klebsiella* type specific polysaccharides or buffer for control. The upper left plate is the control plate which shows a large peak due to precipitation of the monoclonal IgM by anti-μ antiserum. The gels were selected so that the macroglobulin had a slight cathodic mobility in the system employed. In the upper right plate, polysaccharide 12 (PS12) was incorporated into the intermediate gel. Under the conditions employed, PS12 has a slightly anodic mobility and it would therefore meet the macroglobulin which moves towards the cathode. The entire macroglobulin peak has disappeared in this plate, which proves that all of the monoclonal macroglobulin reacts with polysaccharide 12. The lower left plate shows a similar experiment with PS13 incorporated into the intermediate gel. The entire macroglobulin peak disappeared in this plate too, which demonstrates that all of the monoclonal IgM also reacts with polysaccharide 13. The lower right plate is another control where PS35 was incorporated into the gel. IgM Ro did not precipitate with PS35 by double diffusion tests in agarose (Fig. 1) and did not agglutinate red cells coated with PS35 (Table 1). In this experiment, the macroglobulin peak was developed and the area of the precipitate was not significantly different from that of the control plate with buffer in the intermediate gel.

In similar tests with serum We, a large macroglobulin peak was again observed in the control plate with buffer in the intermediate gel, and the entire peak disappeared when PS35 was incorporated into the intermediate gel.

The macroglobulin of serum Th penetrates poorly into agarose gel (cf. Fig. 1), and we did not obtain a good macroglobulin peak by crossed immunoelectrophoresis of this serum. In this case, experiments were then made as for construction of a quantitative precipitin curve using a constant amount of serum Th in each tube and increasing amounts of PS13. The precipitates were spun down and the

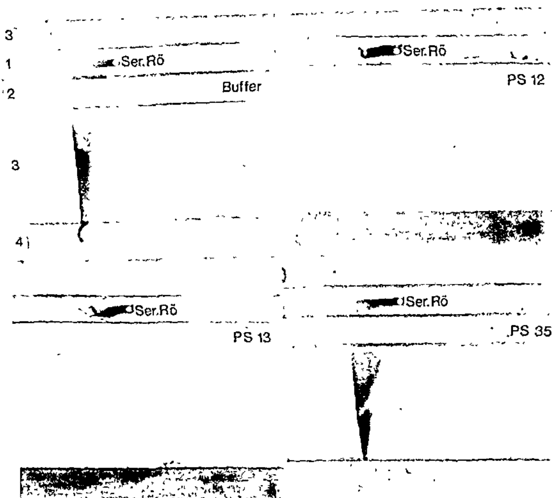


Fig 2. Crossed immunoelectrophoresis of serum Rø in agarose gel containing anti-μ with intermediate gel containing *Klebsiella* type specific polysaccharides. To illustrate that the whole macroglobulin peak is absorbed by PS12 and PS13 whereas it is not affected by PS35.

1· Gel for first dimension electrophoresis, anode to the right.

2 Intermediate gel with buffer (control) or polysaccharide as indicated.

3 and 4 Gels with anti-μ. Gels No. 1, 2 and 3 with agarose with slight endosmotic flow (Litex, batch No. 115). Gel No. 4 with agarose with negligible endosmotic flow (Litex, batch No. 122). Second dimension electrophoresis with anode to the top. For further explanation, see text.

supernatants tested by electrophoresis in agarose gel (Litex, batch No. 115). A typical result is illustrated in Fig. 3. The control electrophoresis of serum Th revealed a dense precipitate at the application site due to the macroglobulin which did not penetrate into the gel. The supernatant after addition of PS13 in antigen excess gave no precipitate at the application site, which shows that the entire macroglobulin band was removed by this procedure.

Based on these experiments, it was concluded that the monoclonal macroglobulins in these three sera interacted specifically with the acid *Klebsiella* type specific polysaccharides and that all of the monoclonal macroglobulin in each serum was involved in this reaction.

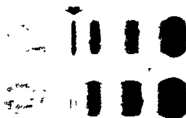


Fig. 3. Electrophoresis of macroglobulinaemia serum Th in agar gel before and after addition of *Klebsiella* type 35 specific polysaccharide. The upper part shows the control; the monoclonal IgM does not penetrate into the gel and gives a marked band at the application site (arrow). The lower part shows electrophoresis on the same plate of the supernatant fluid after addition of excess PS35 to the serum and centrifugation. The monoclonal band has disappeared and there is no significant change of the other protein bands.

Localization of the Binding Site within the IgM Molecule

Metzger (19) has defined a set of criteria which must be fulfilled to accept the reaction between a monoclonal IgM and its ligand as an antigen-antibody reaction. An important requirement is that the binding sites in the IgM molecule must be demonstrated in the Fabμ fragment. For monoclonal IgM interacting with type specific *Klebsiella* polysaccharides, this requirement has previously (10, 11) been fulfilled for IgM To and Næ where sedimentation equilibrium centrifugation of the Fabμ fragment showed entirely different apparent molecular weights in the presence and absence of the type specific polysaccharide with which the protein interacted in gel diffusion tests. Macroglobulins Ro and Th were available in insufficient amounts for direct demonstration of the binding sites in the Fabμ fragment. For IgM We this was demonstrated by enzymatic splitting of IgM and subsequent crossed immunoelectrophoresis with intermediate gel containing PS13, or PS35 for control. Splitting with trypsin gave a fast moving Fcμ fragment which precipitated with anti-μ but not with anti-light chain antisera. In this case, the intermediate gel was placed on the anodic side of the gel used for first dimension electrophoresis so that the Fcμ fragment during second dimension electrophoresis would pass through the area containing polysaccharide before it penetrated into the anti-μ containing gel. The Fcμ fragment penetrated into and gave a similar precipitate in the anti-μ containing gel irrespective of whether PS13 or PS35 were present in the intermediate gel, indicating that it was not bound to the polysaccharides. The Fabμ fragment obtained by pepsin splitting precipitated only with anti-λ. Its slightly cathodic mobility similar to that of the anti-λ antibody in the gel resulted in only faint precipitate formation in the controls with buffer or the nonreactive PS35 in the intermediate gel. Under the conditions employed, PS13 had a slight anodic mobility. When PS13 was incorporated in the intermediate gel placed at the cathodic side of the gel used for first dimension electrophoresis, the Fabμ fragment gave a marked precipitate in the anti-λ containing gel placed on the anodic side of the first dimension gel. The electrophoretic mobility of the Fabμ fragment was thus changed by exposure to PS13 but not by PS35. These findings show that the Fabμ, but not the Fcμ fragment of IgM We is bound to the type specific polysaccharide.

Capsular Swelling Reactions

Fig. 4 shows a typical example of capsular swelling reactions using *Klebsiella* bacteria of different types and macroglobulinaemia serum Ro. Macroglobulinaemia serum Ro induced typical capsular swelling of *Klebsiella* types 12 and 13 whereas it did not induce capsular swelling of *Klebsiella* type 35. This corresponds to its specificity in precipitation and passive haemagglutination tests (cf. Fig. 1 and Table 1).

Table 2 summarizes the results of capsular swelling reactions with the macroglobulinaemia sera and control rabbit immune antisera. The capsular swelling reaction with the macroglobulinaemia sera showed a clear specificity similar to that observed in precipitation and passive haemagglutination tests, and

Klebsiella polysaccharides PS12, PS13 and PS35 (cf. Fig. 1). The present work further demonstrates that the same sera agglutinated red cells coated with these polysaccharides by the chromium chloride technique (5) to give a test system of much higher sensitivity and still showing the same specificity (cf. Table 1). This test system should prove very valuable for further studies of these interactions between monoclonal macroglobulins and *Klebsiella* type specific polysaccharides. It is further demonstrated in the present work that these macroglobulinaemia sera induced typical capsular swelling of *Klebsiella* bacteria, again with the same specificity as in the precipitin and passive haemagglutination tests.

The capsular swelling reaction is probably due to an antigen-antibody reaction on the surface of the capsule of the bacteria which makes the outline of the capsule directly visible in the microscope. The widely used capsular swelling reaction is based on experiments with hyperimmune sera and we have found no specific information on the ability of IgM antibodies to induce capsular swelling. The present investigation clearly shows that monoclonal IgM proteins which interact in a highly specific way with type specific acid polysaccharides of *Klebsiella* also induce capsular swelling. The activity in these sera was confined to the macroglobulin fraction after density gradient ultracentrifugation, and the activity was very strong, in fact much stronger than the activity in the hyperimmune rabbit antisera used as controls.

The observation that macroglobulinaemia serum Ro interacts both with *Klebsiella* type 12 and 13 polysaccharides in precipitin, passive haemagglutination, and capsular swelling reactions is particularly interesting. Cross-reactions between *Klebsiella* type 12 and 13 polysaccharides have never been demonstrated by capsular swelling, neither by us nor in other laboratories (3, 16). A central question is then whether the same IgM molecules interact with the two type specific polysaccharides or whether separate molecules interact with type 12 and the type 13 polysaccharide. The crossed immunoelectrophoresis experiments illustrated in Fig. 2 clearly indicate that all of the monoclonal IgM interacted with polysaccharide 12 and that all of the monoclonal IgM interacted with polysaccharide 13, that is both activities must be present on the same molecule. This would then indicate a different specificity in this monoclonal IgM than in the immune antisera regularly used for capsular swelling reactions. This observation prompted further investigations (6) to establish with other techniques that the same IgM molecule would interact with both polysaccharides, and further to compare the specificity of IgM and IgG antibodies in immune sera against the type 12 and type 13 polysaccharides to see whether cross-reactions might be demonstrated in immune IgM antibodies in contrast to the regular absence of cross-reactions in the IgG antibodies studied until now.

The work was supported by grants from *Anders Jahre's Fund for the Promotion of Science*, the *Norwegian Council for Cardiovascular Diseases*, and the *Norwegian Research Council for Science and the Humanities*.

REFERENCES

1. *Ashman, R. F. & Metzger, H.*: A Waldenström macroglobulin which binds nitrophenyl ligands. J. Biol. Chem. *244:* 3405–3414, 1969.
2. *Axelsen, N. H.:* Intermediate gel in crossed and in fused rocket immunoelectrophoresis. Scand. J. Immunol. *2:* Suppl I, 71–77, 1973.
3. *Edwards, P. R. & Fife, M. A.:* Capsule types of Klebsiella. J. Infect. Dis. *91* 92–104, 1952
4. *Eriksen, J.:* Immunochemical studies on some serological cross-reactions in the *Klebsiella* group. 10. Structure of the capsular polysaccharide of *Klebsiella* type 3(C). Acta path. microbiol. scand. *64:* 347–361, 1965.
5. *Eriksen, J.:* Passive hemagglutination test, using erythrocytes sensitized with Klebsiella type specific polysaccharides Acta path. microbiol. scand. Sect. B, *81:* 309–313, 1973.
6. *Eriksen, J., Harboe, M. & Deverill, J.:* A monoclonal IgM with specific antibody reactivity against *Klebsiella* sero-types 12 *and* 13. Acta path. microbiol. scand. Sect. C, *83:* 105–111, 1975.

7. *Fudenberg, H. H. & Kunkel, H. G.:* Physical properties of the red cell agglutinins in acquired hemolytic anemia. J. Exp. Med. *106:* 689–702, 1957.
8. *Hannestad, K.:* Monoclonal and polyclonal γM rheumatoid factors with anti-di- and anti-trinitrophenol activity. Clin. Exp. Immunol. *4:* 555–578, 1969.
9. *Hannestad, K., Eriksen, J., Christensen, T. & Harboe, M.:* Multiple M-components in a single individual—I. The structural relationship of two serum γMk M-components as revealed by combining specificity and individual antigenic specificity. *Immunochemistry 7:* 899–915, 1970.
10. *Hannestad, K. & Christensen, T. B.:* Multiple M-components in a single individual—II. Studies on urinary Fabμ like fragments in macroglobulinemia. Immunochemistry *8:* 917–931, 1971.
11. *Hannestad, K. & Sletten, K.:* Multiple M-components in a single individual—III. Heterogeneity of M-components in two macroglobulinemia sera with anti-polysaccharide activity. J. Biol. Chem. 246: 6982–6990, 1971.
12. *Harboe, M. & Torsvik, H.:* Protein abnormalities in the cold haemagglutinin syndrome. Scand. J. Haemat. *6:* 416–426, 1969.
13. *Harboe, M. & Fölling, I.:* Complex formation between monoclonal IgM and albumin. Scand. J. Immunol. *3:* 51–60, 1974.
14. *Harboe, M. & Fölling, I.:* Recognition of two distinct groups of human IgM and IgA based on different binding to Staphylococci. Scand. J. Immunol. *3:* 471–482, 1974.
15. *Hauptman, S. & Tomasi, T. B. Jr.:* A monoclonal IgM protein with antibody-like activity for human albumin. J. Clin. Invest. *53:* 932–940, 1974.
16. *Henriksen, S. D.:* Studies on the Klebsiella group (Kauffmann) I. Serotypes of a collection of strains from human sources and from water. Acta path. microbiol. scand. *34:* 249–258, 1954.
17. *Joseph, K. C., Alkin, C. R. & Wistar, R.:* Forssman-containing liposomes: complement-dependent damage due to interaction with a monoclonal IgM. J. Immunol. *112:* 1949–1951, 1974.
18. *Kritzman, J., Kunkel, H. G., McCarthy, J. & Mellors, R. C.:* Studies of a Waldenström-type macroglobulin with rheumatoid factor properties. J. Lab. clin. Med. *57:* 905–917, 1961.
19. *Metzger, H.:* Characterization of a human macroglobulin, V. A Waldenström macroglobulin with antibody activity. Proc. Nat. Acad. Sci. *57:* 1490–1497, 1967.
20. *Solheim, B. G.:* Kinetics of the reaction between a monoclonal IgM anti-I and rabbit red cells. II. Activity of subunits and Fabμ-fragments. Scand. J. Immunol. *1:* 231–236, 1972.
21. *Tolleshaug, H. & Hannestad, K.:* Binding of ligands to a monoclonal IgM macroglobulin with double specificity. Immunochemistry. In press.

Acta path. microbiol. scand. Sect. C, *83:* 106–111, 1975

A MONOCLONAL IgM WITH SPECIFIC ANTIBODY REACTIVITY AGAINST *KLEBSIELLA* SERO-TYPES 12 *AND* 13

JORUNN ERIKSEN, MORTEN HARBOE and JOHANNA DEVERILL

University of Oslo, Kaptein W. Wilhelmsen og Frues Bakteriologiske Institutt, Rikshospitalet, Oslo 1 and Institute for Experimental Medical Research, Ullevål Hospital, Oslo 1, Norway

Eriksen, J., Harboe, M. & Deverill, J. A monoclonal IgM with specific antibody reactivity against *Klebsiella* sero-types 12 *and* 13. Acta path. microbiol. scand. Sect. C, *83:* 106–111, 1975.

Monoclonal IgM from the human macroglobulinaemia serum Rø showed antibody activity against type specific polysaccharide from *Klebsiella* type 12 *and* type 13. The same IgM-molecule precipitated both polysaccharides. Cross-reaction between these two *Klebsiella* types has not been reported previously. It could also be demonstrated with rabbit hyperimmune sera by the passive haemagglutination reaction, but not by gel precipitation or capsular swelling.

Key words: Monoclonal IgM; *Klebsiella* sero-types.

J. Eriksen, Kapt. W. Wilhelmsen og Frues Bakteriologiske Institutt, University of Oslo, Rikshospitalet, Oslo 1, Norway.

Received 3.ix.74 Accepted 15.x.74

Screening of human monoclonal IgM proteins has revealed that they often react with antigens. By many criteria, these IgM proteins behave as specific antibodies. Among 116 sera which contained monoclonal IgM, ten were found to give strong precipitin reactions with capsular polysaccharides isolated from 17 different *Klebsiella* types (5).

In a preceding work (6), three sera containing monoclonal IgM were examined further. There sera, Rø, We and Th were selected because they showed highly specific precipitin reactions with *Klebsiella* type 12, 13 and 35 polysaccharides, respectively. The same specificity was observed in more sensitive passive haemagglutination tests. The activity was confined to the monoclonal IgM present in the sera, and all of the monoclonal IgM in each serum had the characteristic activity.

The capsular swelling reaction is a specific reaction between capsular bacteria and type specific antibody. Serum Rø, We and Th induced capsular swelling of suspensions of *Klebsiella* type 12, 13 and 35 bacteria and, once again, the activity was due to the monoclonal IgM.

But the most interesting observation was the fact that the monoclonal IgM in serum Rø precipitated the type specific polysaccharides both of *Klebsiella* type 12 *and* type 13. The reaction was confirmed by passive haemagglutination and by capsular swelling. Absorption experiments in crossed immunoelectrophoresis showed that the same IgM molecules reacted with type 12 and type 13 polysaccharide.

The purpose of the present work was to confirm this observation by further examinations of serum Rø and to re-examine the cross-reactivity between *Klebsiella* type 12 and type 13 with rabbit immune sera.

MATERIALS AND METHODS

Sera. Serum Rø and serum We containing monoclonal IgM were the same as those reported in the previous paper (6). Serum Rø reacted with *Klebsiella* types 12 and 13, while serum We reacted only with type 13.

Old rabbit sera which had been used as *Klebsiella* typing antisera before, were kindly placed at our disposal by professor *S. D. Henriksen*. These sera were:

Anti-Klebsiella:

type 12 serum from rabbit 50/52,
type 12 serum from rabbit 50/52, 12/6
type 13 serum from rabbit 15/3.52 and
type 13 serum from rabbit 1/4.52

In addition, two recently produced rabbit antisera against each type were examined in the same way. The sera were:

Anti-*Klebsiella*.

type 12, serum 343 from rabbit 31,
type 12, serum 344 from rabbit 32,
type 13, serum 346 from rabbit 34 and
type 13, serum 341 from rabbit 35.

Immunization. Hyperimmune rabbit sera against *Klebsiella* type 12 and 13 were obtained by immunization of rabbits intravenously every fifth day, later once a week. The three first doses of antigen, 0.1, 0.2 and 0.3 ml of a 20 h broth culture, contained formalin killed bacteria. Later 0.3 ml volumes of a living culture were given.

Test bleedings were performed and, if the titre against the homologous *Klebsiella* type was satisfactory, the animal was bled from the heart. All sera were inactivated by heating to 56° C for ½ h before use.

Bacteria. Klebsiella types 12, 13 and 35, originally obtained from The State Serum Institute, Copenhagen, were kept freeze dried. A broth culture was used to examine for capsular swelling.

The capsular swelling method has been described earlier (7).

The capsular, type specific polysaccharides of *Klebsiella* type 12 (PS12), 13 (PS13) and 35 (PS35) were isolated as before (4).

Gel diffusion. Double diffusion tests were made as described in the preceding paper (6).

The passive haemagglutination procedure was described recently (3). When hyperimmune rabbit sera were examined, sheep red blood cells (SRBC) were used as carriers of the polysaccharide.

Mixed haemagglutination. Milgrom et al. (9) used mixed agglutination reactions with coated mammalian and alligator red cells to demonstrate that rheumatoid factor activity against human and rabbit IgG some times was found in the same IgM molecules and in other cases in separate IgM molecules. A similar procedure was therefore used to see whether anti-PS12 and anti-PS13 activity was present on the same or separate IgM molecules in serum Rø.

The mixed haemagglutination reactions were performed with human (HRBC) and chicken (CRBC) erythrocytes as antigen carriers. Red blood cells were washed six times in at least 50 volumes of 0.9 per cent NaCl and centrifuged for 10 min at 1500 rev/min. The coating of the RBC was as follows:

50 µl packed, washed RBC
100 µl polysaccharide, 2 mg/ml in 0.9 per cent NaCl
25 µl 0.1 per cent $CrCl_3$ in 0.9 per cent NaCl, made just before use.

The $CrCl_3$ was added during mixing, and the mixture was shaken vigorously for 5 min in the dark. The coated cells were washed three times with liberal amounts of saline and suspended to suitable strength in 0.9 per cent NaCl, i.e. for HRBC, to a volume of 5 ml, and for CRBC to a volume of 3.5 ml.

Serum Rø and We were diluted 1:1000 with 0.2 per cent human serum albumin in saline (HSA). Equal volumes (100 µl) of serum dilution and coated RBC suspensions were mixed. The mixtures were shaken in the dark for several minutes and left overnight at 4° C. Agglutination was first read macroscopically. If positive, the agglutinates were examined microscopically.

EXPERIMENTS AND RESULTS

To confirm that the monoclonal IgM in serum Rø reacted specifically with both *Klebsiella* type 12 and type 13 polysaccharides, further experiments were performed.

Serum Rø was precipitated with a solution of PS12 dissolved in phosphate buffered saline (PBS) with moderate excess of polysaccharide. The precipitate was washed 4 times with PBS and dissolved in 0.1 M barbiturate buffer of pH 8.6. This procedure was based on the features previously observed in gel diffusion experiments where IgM Rø precipitated type 12 polysaccharide in gels

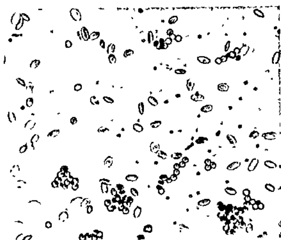


Fig. 1. Agglutination induced by serum Rø when HRBC's are coated with PS12 and CRBC's uncoated.

containing PBS, but not in gels with barbiturate buffer of pH 8.6 (6). 10–40 per cent sucrose solutions were prepared in the latter buffer and, by sucrose density gradient ultracentrifugation, the monoclonal IgM fraction from the precipitate was isolated. The isolated IgM protein was dialysed extensively against PBS and then tested by double diffusion in gel containing PBS against PS12, PS13 and PS35. This specifically isolated IgM precipitated both type 12 and type 13 polysaccharide. This experiment thus confirmed that the monoclonal IgM protein in

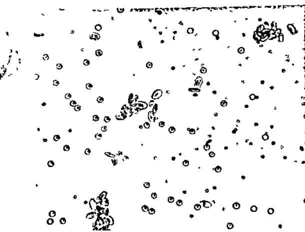


Fig. 2. Agglutination induced by serum Rø when HRBC's are uncoated and CRBC's coated with PS13.



Fig. 3. Agglutination induced by serum Rø when HRBC's are coated with PS12 and CRBC's with PS13.

serum Rø reacted with the polysaccharides 12 *and* 13. No reaction with polysaccharide type 35 could be observed.

This was also demonstrated by mixed haemagglutination. In previous examinations (6), serum Rø showed positive haemagglutination when erythrocytes were coated with either PS12 or PS13. When HRBC's were coated with PS12 and CRBC's with PS13, mixed haemagglutination of both antigens by serum Rø could be observed by microscopic examination of the agglutinates (cf. Table 1 and Fig. 3). In a series of experiments, mixed agglutinates were observed when IgM Rø reacted with two cell types coated with different polysaccharides (cf. Fig. 3). When only one cell type was coated, agglutinates of this type were found among free cells of the other species (cf. Figs. 1 and 2)

These observations are as expected if anti-PS12 and anti-PS13 activity reside in the same IgM molecules in serum Rø. The finding that monoclonal IgM from serum Rø reacted with polysaccharides both from *Klebsiella* type 12 *and* type 13 indicates that these two type specific polysaccharides have common antigenic determinants. The reason why no cross-reactions between the two *Klebsiella* types mentioned were detected in earlier investigations could be that the methods used were not sufficiently sensitive or

TABLE 1. *Mixed Haemagglutination Experiment to Illustrate the Specificity of the System*

	Human red blood cells (HRBC) coated with			Chicken red blood cells (CRBC) coated with			Haemagglutination	
	PS12	PS13	control	PS12	PS13	control	macro-scopically	microscopically with cell content of agglutinates
100 µl 0.2 % HSA mixed with:	100 µl						–	–
		100 µl					–	–
				100 µl			–	–
					100 µl		–	–
100 µl serum Ro diluted 1:1000 in 0.2 % HSA mixed with:	50 µl					50 µl	+ + +	+ + +, HRBC only, Fig. 1
			50 µl		50 µl		+ + +	+ + +, CRBC only, Fig. 2
	50 µl				50 µl		+ + +	+ + +, mixed agglutinates, Fig. 3
100 µl serum We diluted 1:1000 in 0.2 % HSA mixed with:	50 µl				50 µl		+ + +	+ + +, CRBC only
		50 µl		50 µl			+ + +	+ + +, HRBC only
	50 µl					50 µl	–	–
		50 µl				50 µl	+ + +	+ + +, HRBC only

TABLE 2. *Gel Diffusion, Capsular Swelling, and Passive Haemagglutination with Rabbit Immune Sera*

Anti-*Klebsiella* sera against	Gel diffusion against		Capsular swelling of *Klebsiella*		Passive haemagglutination of SRBC coated with		
	PS12	PS13	type 12	type 13	PS12	PS13	uncoated
type 12 from rabbit 50/52	+	–	8*	–§	8192	64	8
type 12 from rabbit 50/52, 12/6	+	–	16	–	8192	128	8
type 12 from rabbit 31, s. 343	+	–	8	–	512	32	4
type 12 from rabbit 32, s. 344	+	–	8	–	1024	64	8
type 13 from rabbit 16/3.52	–	+	–	128	2048	4096	2
type 13 from rabbit 1/4 52	–	+	–	32	256	4096	–
type 13 from rabbit 34, s. 346	–	+	–	256	1024	4096	–
type 13 from rabbit 35, s. 341	–	+	–	64	2048	8192	–

* highest serum dilution giving positive results
§ negative in undiluted serum.

that other types of immune sera should be tested.

Four old rabbit antisera, used earlier as *Klebsiella* typing antisera, and two recently produced rabbit antisera against each type were examined by gel diffusion, capsular swelling and passive haemagglutination. The results are recorded in Table 2.

The results in the table indicate a cross-reaction between *Klebsiella* type 12 and type 13 when passive haemagglutination was used for the examination, as opposed to the results obtained by the two other methods.

DISCUSSION

In the previous paper (6) it was concluded that the monoclonal IgM protein in serum Ro reacted with the specific polysaccharide from *Klebsiella* type 12 as well as type 13, and that the same IgM molecule reacted with both polysaccharides. The latter assumption was further corroborated by the two first experiments described here: IgM Ro isolated from a precipitate produced by reaction with PS12, was shown to be re-precipitated by PS13. This was also con-

firmed by mixed haemagglutination with serum Ro: If human- and chicken erythrocytes were used as carriers of PS12 and PS13, respectively, mixed agglutinates were obtained. If the specificity of monoclonal IgM proteins is similar to that of antibodies induced by immunization, all experiments pointed to the conclusion that the specific polysaccharides of *Klebsiella* types 12 and 13 must have antigenic determinants in common, in other words that the two types cross-react.

In earlier examinations of the *Klebsiella* group, no clear cross-reaction between types 12 and 13 was recorded. According to a report on an examination carried out by *F. Kauffmann* in 1949 (8), tube agglutination was found to bring about a weak cross-reaction. This reaction was not confirmed by capsular swelling reaction (7) even though the *Klebsiella* strains used were the same as those used in Kauffmanns work. In 1952 *Edwards* & *Fife* (1) also studied the capsular types of *Klebsiella* and using both tube agglutination and capsular swelling reaction, they did not find any cross-reaction between the types.

Our results obtained by the capsular swelling reaction (Table 2) were in accordance with earlier examinations. Similar results were obtained by gel diffusion methods. If passive haemagglutination was used, a cross-reaction between the two types could be demonstrated, both with rabbit antisera produced for the present work and with the old type specific antisera. The passive haemagglutination method is more sensitive, compared with capsular swelling and gel precipitation. Specific antisera against *Klebsiella* type 12 agglutinated to some extent SRBC coated with PS13. Still, in antisera against *Klebsiella* type 13, the cross-reaction with PS12 was much stronger.

This is in contrast to the monoclonal IgM in serum Ro which reacted to the same extent with both polysaccharides. The same IgM molecule reacted with both polysaccharides, i.e. with the common antigenic determinant.

The *Klebsiella* antisera used were from hyperimmunized rabbits. As regards nos. 31, 32, and 34 this means 15 immunizations, while rabbit no. 35 got 12. In the case of the old antisera, any information about the number of immunizations is not available. Early after immunization the antibodies are heterogeneous and of the IgM class. By prolonged stimulation of the rabbits with *Klebsiella*, antibodies of restricted heterogeneity are produced (2). It could be of interest to study the specificity of the antibodies during continuous immunization. In many instances, observations initially made on monoclonal immunoglobulins have later led to similar findings in "normal", heterogeneous immunoglobulins. According to the observations on IgM Ro and the presently described rabbit immune sera, one would expect the cross-reaction between *Klebsiella* type 12 and type 13 polysaccharides to be most easily demonstrable in IgM antibodies early after immunization. Experiments to test this hypothesis will be reported in a subsequent paper.

The work was supported by grants from *Anders Jahre's Fund for the Promotion of Science*, the *Norwegian Council for Cardiovascular Diseases*, and the *Norwegian Research Council for Science and the Humanities*.

REFERENCES

1. *Edwards, P. R.* & *Fife, M. A.* Capsule types of *Klebsiella*. J. Infect. Dis. *91*. 92–104, 1952.
2. *Eriksen, J.* & *Haug, R. H.*: Antibodies with restricted electrophoretic heterogeneity in rabbits hyperimmunized with *Klebsiella pneumoniae*. Acta path. microbiol. scand. Sect. B, *82*: 429–438, 1974.
3. *Eriksen, J.*: Passive hemagglutination test, using erythrocytes sensitized with *Klebsiella* type specific polysaccharides. Acta path. microbiol. scand. Sect. B, *81*: 309–313, 1973.
4. *Eriksen, J.*: Immunochemical studies on some serological cross-reactions in the *Klebsiella* group. 10. Structure of the capsular polysaccharide of *Klebsiella* type 3(C). Acta path microbiol. scand. *64*: 347–361, 1965.
5. *Harboe, M.*: Unpublished observations.
6. *Harboe, M.*, *Deverill, J.* & *Eriksen, J.*: Capsular swelling and passive haemagglutination induced by monoclonal IgM reacting with

acid polysaccharides of *Klebsiella*. Acta path. microbiol. scand. Sect. C, *83:* 97–105, 1975.

7. *Henriksen, S. D.:* Studies on the *Klebsiella* group (Kauffmann) 1. Serotypes of a collection of strains from human sources and from water. Acta path. microbiol. scand. *34:* 249–258, 1954.
8. *Kauffmann, F.:* On the serology of the Klebsiella group. Acta path. microbiol. scand. 26: 381–406, 1949.
9. *Milgrom, F., Witebsky, E., Goldstein, R. & Loza, U.:* Studies on the rheumatoid and related serum factors. II. Relation of anti-human and anti-rabbit gamma globulin factors in rheumatoid arthritis serums. J. amer. med. Assoc. *181:* 476–484, 1962.

firmed by mixed haemagglutination with serum Ro: If human- and chicken erythrocytes were used as carriers of PS12 and PS13, respectively, mixed agglutinates were obtained. If the specificity of monoclonal IgM proteins is similar to that of antibodies induced by immunization, all experiments pointed to the conclusion that the specific polysaccharides of *Klebsiella* types 12 and 13 must have antigenic determinants in common, in other words that the two types cross-react.

In earlier examinations of the *Klebsiella* group, no clear cross-reaction between types 12 and 13 was recorded. According to a report on an examination carried out by *F. Kauffmann* in 1949 (8), tube agglutination was found to bring about a weak cross-reaction. This reaction was not confirmed by capsular swelling reaction (7) even though the *Klebsiella* strains used were the same as those used in Kauffmanns work. In 1952 *Edwards & Fife* (1) also studied the capsular types of *Klebsiella* and using both tube agglutination and capsular swelling reaction, they did not find any cross-reaction between the types.

Our results obtained by the capsular swelling reaction (Table 2) were in accordance with earlier examinations. Similar results were obtained by gel diffusion methods. If passive haemagglutination was used, a cross-reaction between the two types could be demonstrated, both with rabbit antisera produced for the present work and with the old type specific antisera. The passive haemagglutination method is more sensitive, compared with capsular swelling and gel precipitation. Specific antisera against *Klebsiella* type 12 agglutinated to some extent SRBC coated with PS13. Still, in antisera against *Klebsiella* type 13, the cross-reaction with PS12 was much stronger.

This is in contrast to the monoclonal IgM in serum Rø which reacted to the same extent with both polysaccharides. The same IgM molecule reacted with both polysaccharides, i.e. with the common antigenic determinant.

The *Klebsiella* antisera used were from hyperimmunized rabbits. As regards nos. 31, 32, and 34 this means 15 immunizations, while rabbit no. 35 got 12. In the case of the old antisera, any information about the number of immunizations is not available. Early after immunization the antibodies are heterogeneous and of the IgM class. By prolonged stimulation of the rabbits with *Klebsiella*, antibodies of restricted heterogeneity are produced (2). It could be of interest to study the specificity of the antibodies during continuous immunization. In many instances, observations initially made on monoclonal immunoglobulins have later led to similar findings in "normal", heterogeneous immunoglobulins. According to the observations on IgM Ro and the presently described rabbit immune sera, one would expect the cross-reaction between *Klebsiella* type 12 and type 13 polysaccharides to be most easily demonstrable in IgM antibodies early after immunization. Experiments to test this hypothesis will be reported in a subsequent paper.

The work was supported by grants from *Anders Jahre's Fund for the Promotion of Science*, the *Norwegian Council for Cardiovascular Diseases*, and the *Norwegian Research Council for Science and the Humanities*.

REFERENCES

1. *Edwards, P. R. & Fife, M. A.*: Capsule types of *Klebsiella*. J. Infect. Dis. *91:* 92–104, 1952
2. *Eriksen, J. & Haug, R. H*: Antibodies with restricted electrophoretic heterogeneity in rabbits hyperimmunized with *Klebsiella pneumoniae*. Acta path. microbiol. scand. Sect. B, *82:* 429–438, 1974.
3. *Eriksen, J*: Passive hemagglutination test, using erythrocytes sensitized with *Klebsiella* type specific polysaccharides. Acta path. microbiol. scand. Sect. B, *81:* 309–313, 1973.
4. *Eriksen, J.*: Immunochemical studies on some serological cross-reactions in the *Klebsiella* group. 10. Structure of the capsular polysaccharide of *Klebsiella* type 3(C). Acta path. microbiol. scand. *64:* 347–361, 1965.
5. *Harboe, M.*: Unpublished observations.
6. *Harboe, M., Deverill, J. & Eriksen, J.*: Capsular swelling and passive haemagglutination induced by monoclonal IgM reacting with

tioned solid-phase RIA has been compared to an IEOP method by which to test two clinical series for HB_sAg. The present study is a continuation of the evaluation of this RIA technique in tests of a blood donor population. The occurrence of HB_sAg in repeated donations from an "old" registered donor population is compared to the incidence of HB_sAg in "new" nonregistered donors. In a series of "new" donors, IEOP and RIA as screening methods for HB_sAg have been studied. The number of cases of suspected transfusion hepatitis encountered during the last two years and a half before the screening of blood donors for HB_sAg was introduced, has been compared to the number of cases encountered during the following four years. The cases of transfusion hepatitis during the last four years have been analysed as regards incubation time, presence of HB_sAg and antibody.

MATERIALS AND METHODS

Blood donors. Annually about 14,000 blood units are collected in the blood bank of Malmö General Hospital. The stock of donors comprises about 3000, with a gain and a loss of about 400 a year. Since March 1970, all blood donations have been tested for HB_sAg Except for the first few months in the spring of 1970 when gel diffusion tests were performed, the same IEOP technique has been used as the screening test for HB_sAg throughout this study. The frequency of HB_sAg in the stock of "old" donors, who were already registered in March 1 1970, has been investigated by IEOP. All repeated donations, from March 1970 until October 1973, totalling about 51,000, have been tested for HB_sAg by IEOP. The number of "new" donors from the same period of time was 1742. Seventy-nine per cent were men and 70 per cent of the total number were under 30 years of age. The "new" donors were tested for HB_sAg both by IEOP and RIA.

Immunoelectroosmophoresis (IEOP) technique. These tests were performed as previously described (7). The technique was controlled with the "Reference hepatitis B antigen panel no. 2" from the Division of Biologics Standards, NIH, Bethesda, Md, as described in a previous report (8), as well as with a local reference panel.

Radioimmunoassay (RIA) technique. The performance of the Austria-125 tests was done as described by *Ling & Overby* (18) and the confirmatory tests as described by *Sgouris* (24), *Prince et al* (20) and *Hansson & Johnsson* (8).

Passive haemagglutination (PHA) tests. Anti-HB_s were tested for by PHA according to the method described by *Vyas & Shulman* (25). The purified antigen was prepared from a HB_sAg-containing serum by treatment with fluorocarbon followed by two consecutive isopycnic CsCl-gradients and gel fractionation with Sephadex G-200. A serum was considered to contain anti-HB_s if the test was positive in serum dilution 1/8 or more.

Subtyping of HB_sAg positive sera. Subtypes ad and ay of HB_sAg positive sera were determined by spur formations in gel diffusion tests according to *Le Bouvier* (16) or alternatively by IEOP tests using monospecific anti-HB_s/y and anti-HB_s/d sera (11). These antisera were produced by immunizing guinea pigs with purified HB_sAg of subtypes ay and ad. The resulting antisera were absorbed with sera containing HB_sAg of heterologous subtypes to give monospecificity. Sera with antigen titres too low to react if these methods were used were subtyped using a PHA test. The serum was mixed with ten units of monospecific anti-HB_s/y serum and incubated at +4° C overnight. This mixture was tested for anti-HB_s using erythrocytes coated with purified HB_sAg/ay. If the anti-HB_s/y still could be detected, the serum contained HB_sAg/ad. If the antibodies had been consumed, the antigen was of subtype ay. To confirm the result a corresponding test using monospecific anti-HB_s/d serum and erythrocytes coated with purified HB_sAg/ad was performed.

Electron microscopy. Electron microscopical examination of sera for HBAg particles was done according to *Almeida & Waterson* (1).

RESULTS

"Old" stock of blood donors tested for HB_sAg for the first time. When testing for HB_sAg was introduced, the stock of registered blood donors comprised about 3000 persons. When tested with IEOP for the first time one donor (0.03 per cent) was detected HB_sAg positive (Table 1). The antigen was of subtype ad.

Repeated donations. IEOP tests for HB_sAg of about 51,000 repeated donations from the once screened donor stock detected two positive units (0.004 per cent) (Table 1). The antigen content of one of the two units was so low that it could only be detected after staining the electrophoresis plate. Later bleedings from the same donor also confirmed that the amount of HB_sAg in the serum was on the borderline to be detected by

TABLE 1. *Prevalence of HB$_s$Ag in the Blood Donor Population of Malmö General Hospital, 1970-1973*

		Test method	No. of HB$_s$Ag-pos.	Subtype
Donors registered before March 1 1970, tested for the first time	∾ 3000	IEOP*	1 (0.03 %)	ad
Repeated donations	∾ 51,000	IEOP	2 (0.004 %)	1 ad 1 ay
Prospective donors	1742	IEOP IEOP + RIA§	8 (0.5 %) 10 (0.6 %)	8 ad †

* *Immunoelectroosmophoresis.*
§ Radioimmunoassay.
† The two additional positive samples not subtyped.

IEOP. By PHA tests it was shown to be of subtype ad. All samples drawn from this donor were positive by RIA. The other positive donor detected was a case of acute hepatitis in late incubation stage. Six days after the blood was drawn, the donor became jaundiced. The HB$_s$Ag was of subtype ay.

Prospective blood donors. Among the 1742 prospective donors registered from March 1970 until October 1973, eight positives (0.5 per cent) were detected by the IEOP method and they could all be identified as HB$_s$Ag/ad (Table 1).

Figure 1 illustrates the result of RIA tests of the prospective blood donors who were HB$_s$Ag negative by IEOP. Out of the 1734 sera, 1722 had count rates below five and the remaining twelve between five and 22 SD above the mean of negative sera. Two sera with count rates of six and 22 SD, respectively, above the mean of negative sera could be neutralized by human anti-HB$_s$ serum but not by normal guinea pig serum i.e. were specific for HB$_s$Ag. Seven sera which in repeated RIA tests gave count rates ranging from five to eight SD above the mean value of negative sera could be neutralized by normal guinea pig serum but not by human anti-HB$_s$ serum. Two sera with count rates of 16 and 18 SD above the mean of negative sera could neither be neutralized by human anti-HB$_s$ serum nor by normal guinea pig serum. HBAg particles could not be found by electron microscopical examination of these two sera. One serum with count rate of nine SD above the mean of negative sera was completely used up and a neutralization test could not be performed. Consequently, only two of the twelve primarily RIA positive sera could be identified as containing HB$_s$Ag. The total number of HB$_s$Ag positives in this series of prospective blood donors increased from eight (0.5 per cent) detected by IEOP to ten (0.6 per cent) detected by RIA. The annual distribution of the ten positive findings is shown in Table 2.

In Table 3, the twelve prospective donors preliminarily found to be positive by the RIA technique have been grouped according to the results of the neutralization tests and follow-up of the recipients. The donor sera of group C could be neutralized by normal guinea pig serum, but not by human anti-HB$_s$ serum. Blood from these donors was given to 36 patients who could be followed for more than six months. No case of transfusion hepatitis was observed among these patients nor in any of the other groups of recipients. The number of patients in the groups A, B and D was too small and/or tl observation times too short to permit ar conclusion concerning the infectivity of the sera.

Cases of transfusion hepatitis. From N vember 1967 until February 1970, 29,5(blood units were transfused. In total, te

TABLE 3. *Follow up Study of 81 Patients who Had Received Blood with Radioimmunoassay Count Rates more than 5 SD Above the Mean of Negative Sera*

	Observation time of recipients <2 months	2–4 months	4–6 months	>6 months	Case of hepatitis
A	3	2	1	1	–
B	1	1		1	–
C	23	8	1	36	–
D	1		2		–

A. Two donors. The sera could be neutralized by human anti-HB_s serum but not by normal guinea pig serum.

B. Two donors. The sera could neither be neutralized by human anti-HB_s serum nor by normal guinea pig serum.

C. Six donors. The sera could be neutralized by *normal guinea pig serum but not by human* anti-HB_s serum.

D. One donor. No identification of the positive radioimmunoassay test was done.

TABLE 4. *Ten Patients in whom Hepatitis Developed within Six Months after they Received Blood Transfusions November 1967–February 1970*

Recipients Patient	Date of transfusion	No. of blood units given to the patient	Interval between transfusions and onset of jaundice (months)
1	November 1967	6	2
2	December 1967	5	4
3	January 1968	1	1¼
4	April 1968	3	4
5	May 1968	8	3
6	June 1968	1	2
7	July 1968	8	4
8	March 1969	4	3
9	July 1969	1	2
10	October 1969	2	3

March 1974), 53,968 blood transfusions were given. Clinical hepatitis developed in six of the transfused patients. This frequency is significantly lower at the level of five per cent (chi square = 4 056). If patients who fell ill less than two months after the blood transfusions are excluded, the difference is still of the same degree of significance. It appears from Table 5, that three of the six cases of hepatitis in the latter series (patients 3, 4 and 5) were negative by IEOP and RIA and lacked anti-HB_s in late convalescent sera. Consequently, these three cases of hepatitis were in all probability not caused by hepatitis B virus. As expected, all blood donations given to these three patients were negative by IEOP. The blood donations given to patients 3 and 4 were negative also if tested retrospectively by RIA. In patient no. 5 the incubation time was very short and a titre rise in complement fixation against cytomegalovirus was significant. The remaining three cases (patients 1, 2 and 6) were all positive for HB_sAg, two by IEOP and one only by RIA. The eight blood units given to patients number 1 and number 2 were only tested by IEOP and not by RIA. The serum of patient number 6 was negative by IEOP but positive by RIA and identity with HB_sAg could be demonstrated by neutralization test.

TABLE 5. *Six Patients in Whom Hepatitis Developed within Six Months after They Received Blood Transfusions March 1970 – March 1973*

Patient	Date of transfusion	HB_sAg		Recipients Subtype of the HB_sAg	Anti-HB_s PHA$	Units of blood given to the patient	Donations HB_sAg		Interval between transfusions and onset of jaundice (months)
		IEOP*	RIA§				IEOP	RIA	
1	March 1970	+	NT"	ay	NT	6	—	NT	3
2	October 1970	+	NT	ad	NT	2	—	NT	4
3	April 1971	—	—		—	5	—	—	1½
4	April 1971	—	—		—	7	—	—†	4
5	April 1973	—	—		—	14	—	NT	¾
6	June 1973	—	+	NT	NT	1	—	—	3

* Immunoelectroosmophoresis.
§ Radioimmunoassay.
$ Passive haemagglutination.
" Not tested.
† Six donations negative, a positive count rate in one case could not be identified as HB_sAg.

The single blood transfusion given to this patient plus one blood sample drawn two months prior to this transfusion and two collected two and three months later from the same donor were all negative for HB_sAg if *tested by IEOP and RIA. Anti-HB_s in the* same serum samples could not be detected. These results would indicate that the hepatitis B infection did not emanate from the transfusion in question. Thus, in total, two out of the six cases might have been caused by blood transfusions containing hepatitis B virus. The donors who gave blood to these two patients were tested during the first six months of this study.

DISCUSSION

The great difference in frequency of HB_sAg among the "new" blood donors (0.5 per *cent) and the stock of blood donors regi*stered at the beginning of the present study (0.03 per cent) illustrates the degree of selec*tion which had been done in the "old" donor* stock on grounds other than HB_sAg-screening before this was introduced in 1970. Having a small, well-known and clinically controlled stock of donors facilitates this selection. The incidence of HB_sAg, found by IEOP (0.5 per cent), among the "new" nonregistered blood donors in Malmö was higher than that recorded in previous Scandinavian reports (4, 10, 13, 19, 21). One explanation of the difference in frequency of HB_sAg in the population of prospective blood donors here reported and in populations discussed in previous works may be that "new" donors and "old" donors comprised in the latter works occasionally have been considered collectively.

Twelve out of the 1734 sera from "new" IEOP negative blood donors gave by RIA count rates of more than five SD above nega*tive controls These twelve donors together* with the eight positive donors found by the IEOP technique gave a total of 20, or 1.2 per cent, positives. This corresponds with the report published by *Ling & Overby* (18) who tested volunteer blood donors by Ausria-125. *In* the present study, only two out of the twelve RIA positive sera could be verified to contain HB_sAg by neutralizing with anti-HB_s serum. This is in accordance with *Prince et al.* (20) who found that only about 20 per cent of RIA positive IEOP negative blood donor sera showed clear-cut neutralization with anti-HB_s serum. None of the 36 patients, observation times covering more than six months, who had received blood from donors with falsely positive RIA values, developed clinical hepatitis (Table 3). This supports the presumption that blood samples from this donor group did not contain infectious hepatitis B virus.

Recently Abbott introduced a much more handy modification of the solid-phase RIA (Ausria II-125). The new test is more rapid and somewhat more sensitive than the old variant. However, also the new test detects *sera with positive count rates* that cannot be neutralized by anti-HB_s sera (9). It is clear that RIA such as the Ausria tests at present *belong among the most sensitive* methods for detecting HB_sAg, but both variants *require* the performance of specificity analysis of all *sera with positive count rates.*

In the stock of "old" blood donors in this study the frequency of HB_sAg was very low when tested by *IEOP. During the first year* of the study (March 1970–February 1971), two cases of HB_sAg positive hepatitis might have been avoided *if all the blood* donations had been tested by RIA. On the other hand, none of the four cases of hepatitis which occurred during *the last three years* among patients who during this period had received about 40,000 blood transfusions, could have been prevented *if Ausria-125 had* been used as the screening test for HB_sAg. The antiserum and the electrophoresis plates are made in our own *laboratory and consequently*, the cost of material used per IEOP test is very *low.* However, in the case of Ausria-125 or Ausria *II-125, the cost is about* 5.50 Swedish crowns (equivalent to $ 1.18) per test. If 50,000 donations had to be tested by Ausria, costs would *have increased* by about 275,000 Swedish crowns. In other words, if all donor

sera had been tested by Ausria-125 during the four years of this study and as a result the two first cases of suspected transfusion hepatitis both had been prevented, costs would have amounted to 140,000 Swedish crowns (equivalent to $ 30,000) per prevented case. Accordingly, the additional sensitivity of RIA is considered too expensive to be of practical value in routine examinations of all donations from a blood donor population like the one studied. It must be borne in mind, however, that if optimal sensitivity of the IEOP technique is to be obtained, the antiserum and gel composition must be carefully selected, the electrophoresis plates stained and the test system should be controlled by a suitable panel (8). It might be mentioned that the present authors have examined several commercial IEOP kits and most of these have not been found sufficiently sensitive for a testing of blood donors for the presence of HB_sAg (9). If the 400–500 "new" donors registered anually were to be tested also by Ausria-125 the number of detected HB_sAg-positives would increase by 0.1 per cent, i.e. one positive case every second year. In other blood donor populations in which the incidence of HB_sAg is higher and the frequency of "new" donors greater, the use of a method more sensitive than IEOP may be more strongly indicated.

Recently *Arndt-Hanser et al.* (3) suggested that HB_sAg testing of blood donors does not reduce the incidence of transfusion hepatitis. It has also been assumed that most of HB_sAg positive blood donors might not be infectious and that routine tests for HB_sAg was not indicated (5, 21, 22). In the present study, however, rejection of HB_sAg positive donors brought about that the number of cases of suspected transfusion hepatitis was reduced, the reduction being significant at the five per cent level. This reduction is in accordance with other reports (2, 12, 23) invalidating the theory that the majority of HB_sAg positive donors might not be infectious. Furthermore, *Gocke* (6) has shown that transfusion of blood containing HB_sAg was followed by development of hepatitis and/or production of HB_sAg or anti-HB_s in 75 per cent of the recipients. It might also be mentioned that blood drawn during the incubation stage of hepatitis B is considered highly contagious (14). In the stock of "old" donors, the present authors have observed two cases where HB_sAg positive blood units had been drawn in the incubation stage of acute hepatitis. One case was found while the study was in progress, the other after it was terminated. Altogether, except for the reports by *Arndt-Hanser et al.* (3) and *Reinicke et al.* (21, 22), there are clear-cut indications that screening for HB_sAg of all blood donations should be performed.

We are indebted to Dr. *A. Rausing* of the Department of Clinical Pathology, Malmö General Hospital, for performing the electron microscopical surveys. Professor *G. Le Bouvier* of Yale University kindly tested reference antigens for subtypes ad and ay. The skilful technical assistance of Mrs. *Zita Serléus* is gratefully acknowledged.

REFERENCES

1. *Almeida, J. D. & Waterson, A. P.*: Immune complexes in hepatitis. Lancet ii: 983–986, 1969.
2. *Alter, H. J., Holland, P. V. & Purcell, R. H.*: Posttransfusion hepatitis after exclusion of commercial and hepatitis-B antigen-positive donors. Ann. intern. Med 77: 691–699, 1972.
3. *Arndt-Hanser, A., Holtermüller, K.-H., Ananthakrishnan, R., Ewe, K., Maass, G., Reinicke, V., Köhler, A., Fiedler, H., Rossi, G. & Vermylen, C.*: Prevention of post-transfusion hepatitis. Lancet ii. 345, 1974.
4. *Berg, K.*: Studies on the Australia-SH antigen in Norway. Vox Sang. 19: 384–390, 1970.
5. *Dybkjaer, E. & Reinicke, V.*: Undersogelser for HA-antigen (Australia-antigen) og beskyttelse mod hepatitissmitte. Ugeskr. Laeg. 134: 2543, 1972.
6. *Gocke, D. J*: A prospective study of post-transfusion hepatitis: the role of Australia antigen. J. Amer med. Ass. 219: 1165–1170, 1972.
7. *Hansson, B. G. & Johnsson, T.*: Improved technique for detecting Australia antigen by immunoelectroosmophoresis. Vox Sang. 21 531–539, 1971.
8. *Hansson, B. G. & Johnsson, T.*: Evaluation of a solid phase radioimmunoassay (Ausria-

125) for the detection of hepatitis B antigen (Australia antigen) in two clinical materials. J. clin. Path. *27,* 35–39, 1974.
9. *Hansson, B. G. & Johnsson, T.:* Unpublished work.
10. *Helske, T.:* Personal communication, 1973.
11. *Holland, P. V., Purcell, R. H., Smith, H. & Alter, H. J.:* Subtyping of hepatitis-associated antigen (HBAg); Simplified technique with counterelectrophoresis. J. Immunol. *109:* 420–425, 1972.
12. *Hollinger, F. B., Werch, J. & Melnick, J. L.:* A prospective study indicating that double-antibody radioimmunoassay reduces the incidence of post-transfusion hepatitis B. New Engl. J. Med. *290:* 1104–1109, 1974.
13. *Iwarson, S., Lindholm, A., Lundin, P. & Hermodsson, S.:* Hepatitis-associated antigen and antibody in Swedish blood donors. Vox. Sang. *22:* 501–509, 1972.
14. *Iwarson, S., Magnius, L., Lindholm, A. & Lundin, P.:* Subtypes of hepatitis B antigen in blood donors and post-transfusion hepatitis: clinical and epidemiological aspects. Brit. med. J. *1:* 84–87, 1973.
15. *Jennings, E. R., De Pratti, V., Monroe, C. H., Pollack, W., Watson-Williams, E. J. & Clauss, B.:* Sensitivity of direct solid-phase radioimmunoassay for detection of hepatitis-associated antigen. Lancet *ii:* 734–735, 1973.
16. *Le Bouvier, G. L.:* The heterogeneity of Australia antigen. J. infect. Dis. *123:* 671–675, 1971.
17. *Leers, W.-D. & Kouroupis, G. M.:* Hepatitis B antigen in screened blood units for transfusion in renal dialysis patients. Canad. med. Ass. J. *110:* 308–312, 1974.
18. *Ling, C. M. & Overby, L. R.:* Prevalence of hepatitis B virus antigen as revealed by direct radioimmune assay with ^{125}I-antibody. J. Immunol. *109:* 834–841, 1972.
19. *Nordenfelt, E.:* Australien-antigen och hepatit. Thesis. Studentlitteratur, Lund 1971, p. 17.
20. *Prince, A. M., Brotman, B., Jass, D. & Ikram, H.:* Specificity of the direct solid-phase radioimmunoassay for detection of hepatitis-B antigen. Lancet *i:* 1346–1350, 1973.
21. *Reinicke, V., Dybkjaer, E., Poulsen, H., Banke, O., Lylloff, K. & Nordenfelt, E.:* A study of Australia-antigen-positive blood donors and their recipients, with special reference to liver histology. New Engl. J. Med. *286:* 867–870, 1972.
22. *Reinicke, V., Banke, O. & Dybkjaer, E.:* Australia antigen and posttransfusion hepatitis. A study of Danish blood donors and recipients. Vox sang. suppl. *24:* 65–71, 1973.
23. *Senior, J. R., Goeser, E., Dahlke, M., London, T., Sutnick, A. & Blumberg, B.:* Reduction in post-transfusion hepatitis after rejection of donor blood containing Australia antigen. Gastroenterology *60:* 752, 1971.
24. *Sgouris, J. T.:* Limitations of the radioimmunoassay for hepatitis B antigen. New Engl. J. Med. *288:* 160–161, 1973.
25. *Vyas, G. N. & Shulman, N. R.:* Hemagglutination assay for antigen and antibody associated with viral hepatitis. Science *170:* 332–333, 1970.

Acta path. microbiol. scand. Sect. C, 83: 121–128, 1975

PHAGOCYTOSIS AND MICROBICIDAL CAPACITY OF MOUSE MACROPHAGES NON-SPECIFICALLY ACTIVATED *IN VITRO*

A. Reikvam* and E. A. Høiby**

Norwegian Defence Microbiological Laboratory, Oslo, Norway

Reikvam, A. & Høiby, E. A. Phagocytosis and microbicidal capacity of mouse macrophages non-specifically activated *in vitro*. Acta path. microbiol. scand. Sect. C, 83: 121–128, 1975.

Mouse macrophages cultured in a medium containing 50 per cent newborn calf serum (NBCS) were shown to undergo a characteristic development. Their size as well as their content of phase-lucent and phase-dense granules increased. The cell content of the lysosomal enzyme acid phosphatase was increased. In contrast, macrophages from the same source and cultured in a medium containing 50 per cent foetal calf serum (FCS) were not activated in this way. NBCS-activated macrophages had a markedly enhanced capacity to phagocytose *Toxoplasma gondii* and *Listeria monocytogenes*, as compared with macrophages cultured in FCS. However, their ability to restrict the intracellular multiplication of these micro-organisms was not markedly increased.

Key words: Mouse macrophages; phagocytosis; microbicidal capacity.

A. Reikvam, Institute of Physiology, University of Oslo, Karl Johans gate 47, Oslo 1, Norway.

Received 14.x.74 Accepted 7.xi.74

According to current views, mononuclear phagocytes are activated *in vivo* by lymphokines secreted from immune lymphocytes. Both marked morphological and metabolic changes then occur (18). The activation is claimed to be non-specific and thus, the mononuclear phagocytes acquire an increased capacity to ingest and digest various micro-organisms, including micro-organisms unrelated to the lymphocyte activator (2).

A non-immunological *in vitro* activation of mononuclear phagocytes has been described by *Cohn* & *Benson* (7). Such activation could be obtained with several different substances (10, 11). However, the most potent stimulator of pinocytosis and formation of lysosomal enzymes was postcolostral newborn calf serum (NBCS) (8). The stimulating property was ascribed to a naturally occurring immunoglobulin of the IgM class present in NBCS. This immunoglobulin could also agglutinate mouse erythrocytes. In contrast, foetal calf serum (FCS) lacked this immunoglobulin and could neither activate macrophages nor agglutinate erythrocytes.

The aim of the present work was to investigate the capacity of *in vitro* activated macrophages to phagocytose intracellular micro-organisms, and furthermore to ascertain whether these macrophages could restrict the intracellular growth of the micro-organisms.

* The work was carried out during leave of absence from Institute of Physiology, University of Oslo.

** Present address: National Institute of Public Health, Oslo.

MATERIALS AND METHODS

Animals. Specific, pathogen-free female mice of the strain NMRI/Bom were used. They were 1–3 months old, and weighed 22–30 g. Repeated testing for occurrence of *Toxoplasma gondii* infection using the Dye Test did not reveal any seropositive mice.

Harvesting and processing of mouse mononuclear cells. Mice were killed with ether and the abdominal skin reflected. The peritoneal cells were harvested by washing out the peritoneum with 1–2 ml of medium 199 (Gibco) containing 50 I.U./ml of crystalline penicillin (Glaxo) and 50 μg/ml of streptomycin (Glaxo) (7). The cells were centrifuged down at $120 \times g$ for 5 minutes and resuspended in medium 199 containing 20–50 per cent inactivated (56° C, 35 minutes) FCS (Gibco) and antibiotics as above. The cells were counted in a haemocytometer and the cell concentration adjusted to $2–3 \times 10^6$ cells/ml. Aliquots of 1 ml of this suspension were distributed in Leighton tubes, each containing a 9×35 mm "flying cover slip", which were incubated for one hour at 37° C in an atmosphere of 5 per cent CO_2 in air. Thereafter the tubes were agitated vigorously. The supernatant fluid was discarded and the glass adherent cells washed once with medium 199. The culture media were then added. The Leighton tubes received medium 199 with antibiotics, half of them with 50 per cent NBCS (Microbiological Associates Inc.), the other half with 50 per cent FCS. The incubation was thereafter re-established. The medium was changed once or twice before the cell cultures were used after 4 days.

Toxoplasma gondii. The RH-strain (*Sabin*[21]) was passaged intraperitoneally at 3-days intervals in NMRI/Bom mice. After 3 days of infection, the peritoneal exudate was harvested by a pasteur pipette and mixed with medium 199 containing antibiotics and heparin (50 I.U./ml, Novo), 4 parts exudate to 1 part medium. To obtain a homogenous *T. gondii* suspension, cells and other aggregates must be removed. This was done by differential centrifugation, using a modification of the method described by *Lycke and Lund* (16). The first step involved centrifugation of $65 \times g$ for 5 minutes The sediment was discarded and the supernatant fluid spun again at $440 \times g$ for 20 minutes The supernatant was discarded and the pellet resuspended in medium 199. This suspension was again centrifuged at $65 \times g$ for 5 minutes. The supernatant thus obtained was used. The latter was a homogeneous suspension of *T. gondii* organisms containing only a few mouse cells. More than 90 per cent of the parasites were viable as judged by methylene blue staining After counting in a haemocytometer the concentration was adjusted to $2–4 \times 10^6$ *T. gondii*/ml. One ml of this suspension was added to each macrophage culture.

Preliminary experiments showed that the growth media used were excellent for keeping *T. gondii* alive and that NBCS and FCS were equally well suited for this purpose.

Listeria monocytogenes. A rough strain of *L. monocytogenes* was used. It was derived from a smooth strain isolated from human spinal fluid and kindly supplied from the Bacteriological Department, National Institute of Public Health, Oslo. To obtain a suitable number of organisms for infection, an 18 hour broth culture was used, diluted in medium 199 and counted by the pour plate technique.

Infection of macrophage monolayers. The number of macrophages remained constant throughout the 4-day-culture periods and the cell number was approximately the same in the NBCS- and the FCS-group. Since *L. monocytogenes* is sensitive to penicillin and streptomycin, the medium containing antibiotics was removed 24 hours before the infection with this bacterium, the cells were washed 3 times with medium 199, and then reincubated with the same type of medium, but now without antibiotics. Each macrophage culture was infected with $2–4 \times 10^6$ *T. gondii* or with 10^7 *L. monocytogenes*. After one hour of infection, the medium was removed and the monolayers washed 2–3 times in order to remove the extracellular micro-organisms After addition of new medium (without antibiotics in the *L monocytogenes* experiments), incubation was resumed.

In one type of experiment, *T. gondii* were left in the supernatant throughout the whole experiment (3 hours). This procedure was chosen in order to examine the phagocytic capacity over a longer period of time.

Evaluation of phagocytosis and intracellular fate of the micro-organisms. In each experiment, 16–30 Leighton tubes were set up and groups of 4 tubes were harvested at different times. The cover slips were fixed in methanol and stained with Giemsa. The number of micro-organisms present in 300–500 macrophages randomly chosen on each cover slip was then determined. This technique of quantitation is not very accurate, but sufficiently reliable for detecting marked differences.

Histochemical technique. Acid phosphatase staining was performed using the naphtol AS phosphate method (1).

Microscopy. In order to study intracellular organelles, phase-contrast microscopy was performed on living cells. A plastic frame was glued to a glass slide. Thus shallow troughs were formed After filling the troughs with growth medium, the cover slips were placed over them, the monolayers facing downwards. In this way, the cells could be kept alive for hours and phase-contrast microscopy and photography could be performed, using the 95X oil immersion lens.

Photography. All photographs were taken with a

Leitz Orthomat camera, using a Kodachrome II type A professional film. Magnifications seen on the film were 200 and 500, respectively.

Statistics. A two-sided Wilcoxon-van Eltern rank test was used to evaluate the differences in phagocytic capacity of activated and non-activated macrophages. Ranking was done separately of the results from each period of infection. The sums were weighed, applying the number of cultures in the activated and the control groups. The added values were tested for statistical significance, utilizing a computer program.

RESULTS

Differentiation of macrophages. The macrophages cultured in NCBS and in FCS showed a marked difference. Morphological differences were evident after one day and were accentuated during the following days. NBCS-macrophages increased their content of cytoplasma (Fig. 1 a) whereas FCS-macrophages remained small, although they spread well on the glass (Fig. 1 b).

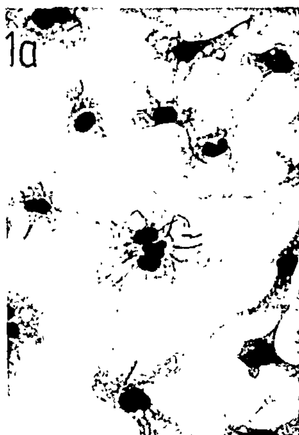
Phase-contrast microscopy revealed that the NBCS-macrophages had a highly vacuolated appearance, probably as a result of increased pinocytosis. They also contained a much higher number of mitochondria as well as phase-lucent and phase-dense granules than the FCS-macrophages. A special characteristic of the NBCS-macrophages was the content of large phase-dense granules in the perinuclear region (Fig. 1 c). These are lysosomes (12). Such large granules were never observed in FCS-macrophages (Fig. 1 d). Acid phosphatase staining showed that NBCS-macrophages stained much more intensely than FCS-macrophages and, characteristically, the staining was most intense in the perinuclear region, the zone occupied by lysosomes.

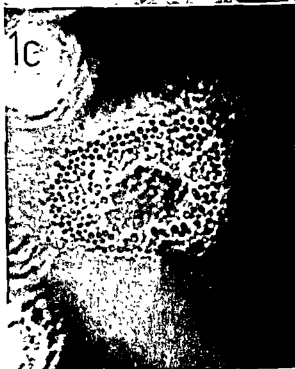
Phagocytosis and fate of intracellular T. gondii. Macrophage monolayers were infected with *T. gondii* and the cell cultures were harvested after 45 minutes, 1½ hour and 3 hours. The number of *T. gondii* left in the supernatant fluid of each Leighton tube was counted in a haemocytometer (Fig. 2). There were fewer *T. gondii* in the NBCS group than in the FCS controls at all intervals. The difference was statistically significant ($p = 0.005$). Since *T. gondii* cannot multiply extracellularly and since there is a lag phase of 8–10 hours before it will start to proliferate intracellularly (15, 19), the data show that NBCS-activated macrophages have an increased phagocytic capacity.

This interpretation was supported by determination of the number of *T. gondii* per cell (infected and non-infected cells included) (Fig. 3). The portion of infected cells was higher in the NBCS-macrophages than in FCS-macrophages and the former group contained also the highest number of protozoa per cell. After 45 minutes, the ratio *T. gondii*/cell in the case of NBCS-macrophages was thus almost twice that observed in the case of FCS-macrophages. After 1½ hour, the same tendency was observed. However, after 3 hours the ratio of the FCS-macrophages had continued to increase and was now slightly higher than that of NBCS-macrophages which had decreased since the 1½ hour measurement.

In another series of experiments, extracellular *T. gondii* were removed by washing the cultures one hour after the addition of the protozoa and the cultures were harvested two hours and 17 hours after the start of the infection (Fig. 4). The results applying to the shorter period were consistent with the results above, showing a markedly increased phagocytosis by the NBCS-macrophages. After 17 hours, some *T. gondii* had multiplied 2–3 times and intracellular colonies, even rosettes of 4 and 8 protozoa, could be observed (Fig. 5). Multiplication was seen in both types of macrophages, being of about the same extent in both types.

Phagocytosis and intracellular fate of Listeria monocytogenes. Macrophage monolayers were infected with *L. monocytogenes* and, after one hour of incubation, extracellular microbes were removed by thoroughly washing 3 times. Cell cultures were harvested after 1, 3, and 5 hours (Fig. 6). The 1-hour results show that NBCS-macrophages had taken up more than twice as many bacteria as the FCS-macrophages. Phagocytosis was


1a

1b

1c

1d

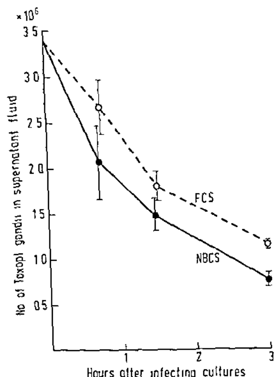


Fig. 2. Phagocytosis of *T. gondii*. Number of *Toxoplasma gondii* left in supernatant fluid after infecting 4 days old macrophage monolayers. Newborn calf serum activated macrophages (NBCS, closed symbols) and macrophages cultured in the presence of foetal calf serum (FCS, open symbols). Each point represents the mean of four cultures ± 1 standard error indicated by vertical bars.

obviously increased. The 3- and 5-hour values show that the number of intracellular bacteria had reached the highest absolute level in the NBCS-group. The relative increase, however, was greatest in the FCS-group. Here the increase in the ratio bacteria/cell in the interval from 1 to 5 hours was 126 per cent, while it was only 51 per cent in the NBCS-group. At 5 hours, some of the macrophages were not adhering to the glass, probably as a result of lysis because of the heavy infection. The number of macrophages had reduced by about 25 per cent in both groups.

DISCUSSION

The present experiments confirmed that NBCS is a potent stimulator of pinocytosis and lysosomal formation *in vitro*, as shown by *Cohn et al.* (8, 9). We found that phagocytosis of two types of intracellular micro-

Fig. 1 a. NBCS-macrophages in 4-day culture. *Listeria monocytogenes* are seen in the cytoplasma. × 640.

Fig. 1 b. Control FCS-macrophages in 4-day-culture. Same magnification as in 1 a

Fig. 1 c. Phase contrast micrograph of a living NBCS-macrophage in 4-day-culture. Note the abundant phase-dense granules which surround the nucleus. × 1800.

Fig. 1 d. FCS-macrophage in 4-day-culture. Conditions as in 1 c.

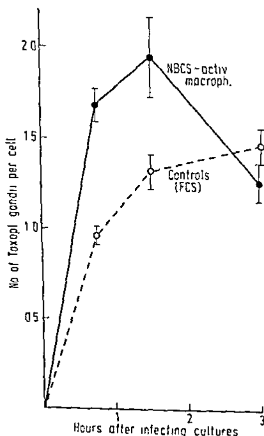


Fig. 3. Phagocytosis of *T. gondii* Same experiment as in Fig. 2. In each culture, the number of *T. gondii* in 300-500 macrophages was counted. Infected and non-infected macrophages included.

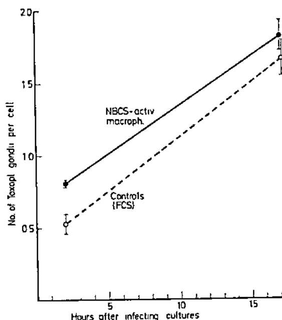


Fig. 4. Fate of *T. gondii* in macrophages. 4-day macrophage monolayers were infected with *T. gondii* for 1 hour. Subsequently, extracellular *T. gondii* were washed away and incubation continued for 1 or 16 hours. In each culture, the number of intracellular *T. gondii* in 500 macrophages were counted. Newborn calf serum activated macrophages (NBCS, closed symbols) and macrophages cultured in the presence of foetal calf serum (FCS, open symbols). The mean with ± 1 standard error of four cultures is shown.

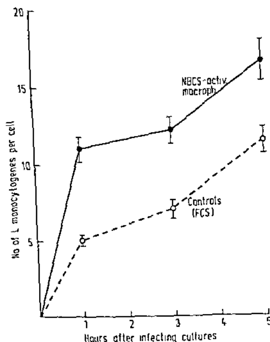


Fig. 5. Macrophage with *Toxoplasma gondii.* At the top, a characteristic rosette.

organisms, i.e. *T. gondii* and *L. monocytogenes* was also markedly stimulated in NBCS-activated macrophages.

The ability to restrict the intracellular growth of the micro-organisms, however, was not markedly increased. The 3-hour results of the phagocytosis experiments with *T. gondii* could indicate an increased killing capacity of the NBCS activated macrophages. The difference, however, may be due to the fact that *T. gondii* present in the supernatant fluid of the NBCS-cultures were fewer than in those of the FCS-cultures at the end of the culture period and thus, the number available for phagocytosis was lower. It must also be kept in mind that even normal, unstimulated macrophages can kill and disintegrate a proportion of the ingested *T. gondii* (13, 14).

The intracellular proliferation of *L. monocytogenes* was lower in the NBCS-macrophages than in the FCS-macrophages, as judged by the percentage increase after the phagocytic step. Since the total number of bacteria was higher in the NBCS-macrophages and since they indeed were heavily infected, however, a saturation phenomenon may have interfered. The interpretation is further complicated because *L. monocytogenes* is capable of extracellular multiplication. It has recently been claimed that it is impossible to wash away all extracellular *L. monocytogenes* and that those remaining extracellularly divide at a rapid rate, the generation time being 45 minutes (24). Such extracellular microbes might be most important for the destruction of the macrophage monolayers (24). In contrast to this rapid extracellular multiplication, *Mackaness* found



ig. 6. Fate of *L. monocytogenes* in macrophages. -day macrophage monolayers were infected with *Listeria monocytogenes* for one hour. Subsequently, ome cultures were harvested; the others were vashed and re-incubated for 3 or 5 hours. Number of *L. monocytogenes* per macrophage is given (300 nacrophages scored). Newborn calf serum activated nacrophages (NBCS, closed symbols) and macrophages cultured in the presence of foetal calf serum (FCS, open symbols). The mean with ± 1 standard error of 4 cultures is shown.

a generation time of 4.8 and 5.1 hours of *L. monocytogenes* growing in mouse spleen and liver cells, respectively (17). In order to avoid extracellular multiplication, the experiments could have been performed with antibiotics added after the washing procedure. But antibiotics are taken up by macrophages (3, 4), probably to the greatest extent in the macrophages with the highest pinocytotic activity. In the present experiment, this would have affected the NBCS-macrophages more than the FCS-macrophages. This type of difficulty was not encountered in the experiments with the obligatory intracellular *T. gondii*.

Most authors believe that lysosomal enzymes are essential in some way or another for the killing and disintegration of micro-organisms (6), but virtually no details are known about the processes involved (5). Macrophages activated immunologically *in vivo* or by lymphokines *in vitro* have an increased content of lysosomes (2, 20) and lysosomal enzymes (20, 22, 23), in similarity with our NBCS-activated macrophages. Consequently, if lysosomal enzymes are critical for the ability to handle micro-organisms, NBCS-macrophages might be expected to be effective in this respect. Our results show, however, that their killing ability was not markedly increased and was much lower than that of macrophages activated *in vivo* by infection (to be published).

The present experiments suggest that factors other than the content of lysosomal enzymes are decisive for the microbicidal capacity exerted by macrophages. In an attempt to obtain further evidence for this viewpoint a direct comparison of *in vivo* and *in vitro* activated macrophages is being undertaken.

The helpful advices of Dr. *Haakon Benestad* and Dr. *Otto Closs* are gratefully acknowledged. The histochemical technique was kindly performed at Department of Pathology, Ullevål Hospital.

REFERENCES

1. *Bancroft, J. D.:* An introduction to histochemical technique. Butterworth & Co. (Publishers) Ltd., London 1967, pp. 192–194.
2. *Blanden, R. V.:* Modification of macrophage function. J. reticulendoth. Soc. *5:* 179–202, 1968.
3. *Bonventre, P. F. & Imhoff, J. G.:* Uptake of ^{3}H-dihydrostreptomycin by macrophages in culture. Infect. Immun. *2:* 89–95, 1970.
4. *Chang, Y. T.:* Suppressive activity of streptomycin on the growth of *Mycobacterium lepraemurium* in macrophage cultures. Appl. Microbiol. *17:* 750–754, 1969.
5. *Cline, M. J.:* Bactericidal activity of human macrophages: analysis of factors influencing the killing of *Listeria monocytogenes*. Infect. Immun. *2:* 156–161, 1970.
6. *Cohn, Z. A., Hirsch, J. G. & Wiener, E.:* Lysosomes and endocytosis. The cytoplasmic granules of phagocytic cells and degradation

of bacteria. In: Reuck, A. V. S. de & Cameron, M. P. (Eds.): Lysosomes. Churchill, London 1963, pp. 126–144.

7. *Cohn, Z. A.* & *Benson, B.:* The differentiation of mononuclear phagocytes. Morphology, cytochemistry and biochemistry. J. exp. Med. *121:* 153–169, 1965 a.

8. *Cohn, Z. A.* & *Benson, B.:* The in vitro differentiation of mononuclear phagocytes. II. The influence of serum on granule formation, hydrolase production, and pinocytosis. J. exp. Med. *121:* 835–848, 1965 b.

9. *Cohn, Z. A., Fedorko, M. E.* & *Hirsch, J. G.:* The in vitro differentiation of mononuclear phagocytes. V. The formation of macrophage lysosomes. J. exp. Med. *123:* 757–766, 1966.

10. *Cohn, Z. A.* & *Parks, E.:* The regulation of pinocytosis in mouse macrophages. II. Factors inducing vesicle formation. J. exp. Med. *125:* 213–237, 1967 a.

11. *Cohn, Z. A.* & *Parks, E.:* The regulation of pinocytosis in mouse macrophages. III. The induction of vesicle formation by nucleosides and nucleotides. J. exp. Med. *125:* 457–473, 1967 b.

12. *Cohn, Z. A.* & *Parks, E.:* The regulation of pinocytosis in mouse macrophages. IV. The immunological induction of pinocytic vesicles, secondary lysosomes, and hydrolytic enzymes. J. exp. Med. *125:* 1091–1111, 1967 c.

13. *Jones, T. C., Yeh, S.* & *Hirsch, J. G.:* The interaction between *Toxoplasma gondii* and mammalian cells. I. Mechanism of entry and intracellular fate of the parasite. J. exp. Med. *136:* 1157–1172, 1972.

14. *Jones, T. C.* & *Hirsch, J. G.:* The interaction between *Toxoplasma gondii* and mammalian cells. II. The absence of lysosomal fusion with phagocytic vacuoles containing living parasites. J. exp. Med. *136:* 1173–1194, 1972.

15. *Kaufman, H. E.* & *Maloney, E. D.:* Multiplication of three strains of *Toxoplasma gondii* in tissue cultures. J. Parasitol. *48:* 358–361, 1962.

16. *Lycke, E.* & *Lund, E.:* A tissue culture method for titration of infectivity and determination of growth rate of *Toxoplasma gondii* I. Acta path. microbiol. scand. *60:* 209–220, 1964.

17. *Mackaness, G. B.:* Cellular resistance to infection. J. exp. Med. *116:* 381–406, 1962.

18. *Mackaness, G. B.:* Cellular immunity. In: Furth, R. van (Ed.): Mononuclear phagocytes. Blackwell Scientific Publications. Oxford 1970, pp. 461–475.

19. *Maloney, E. D.* & *Kaufman, H. E.:* Multiplication and therapy of *Toxoplasma gondii* in tissue cultures. J. Bact. *88:* 319–321, 1964.

20. *Nath, J., Poulter, L. W.* & *Turk, J. L.:* Effect of lymphocyte mediators on macrophages in vitro. A correlation of morphological and cytochemical changes. Clin. exp. Immunol. *13:* 455–466, 1973.

21. *Sabin, A. B.:* Toxoplasmic encephalitis in children. J. Amer. med. Ass. *116:* 801–807, 1941.

22. *Saito, K.* & *Suter, E.:* Lysosomal acid hydrolases in mice infected with BCG. J. exp. Med. *121:* 727–738, 1965.

23. *Suter, E.* & *Hulliger, L.:* Nonspecific and specific cellular reactions to infections. Ann N. Y. Acad. Sci. *88:* 1237–1245, 1960.

24. *Wilder, M. S.* & *Edberg, J. C.:* Interaction of virulent and avirulent *Listeria monocytogenes* with cultured mouse peritoneal macrophages Infect. Immun. *7:* 409–415, 1973

Acta path. microbiol. scand. Sect. C, *83:* 129–138, 1975

ACTIVATED MOUSE MACROPHAGES: MORPHOLOGY, LYSOSOMAL BIOCHEMISTRY, AND MICROBICIDAL PROPERTIES OF *IN VIVO* AND *IN VITRO* ACTIVATED CELLS

A. Reikvam, R. Grammeltvedt* and E. A. Hoiby**

Norwegian Defence Microbiological Laboratory, Oslo, Institute of Physiology, University of Oslo, and Institute of Zoophysiology, University of Oslo, Oslo, Norway

Reikvam, A., Grammeltvedt, R. & Hoiby, E. A. Activated mouse macrophages: Morphology, lysosomal biochemistry, and microbicidal properties of *in vivo* and *in vitro* activated cells. Acta path. microbiol. scand. Sect. C, *83:* 129–138, 1975.

Peritoneal macrophages harvested from mice with a 14 days old Toxoplasma (Beverly strain) infection were compared with normal macrophages activated by culture in a medium containing 50 per cent newborn calf serum (NBCS). Morphologically they seemed to be similar and numerous lysosomes appeared in both types. The lysosomal enzymes acid phosphatase and β-glucuronidase were increased in both macrophage types. Per mg protein, acid phosphatase was increased 6.9 × in *in vitro* activated macrophages and 4.6 × in *in vivo* activated cells, and β-glucuronidase 2.0 × and 2.2 ×, respectively. The capacity of the two cell types to phagocytose the RH-strain of *T. gondii* and a strain of *L. monocytogenes* was approximately identical. In contrast, *in vivo* activated macrophages were much more effective in restricting intracellular growth of the micro-organisms. Mice initially infected with the Beverly strain and then challenged with the RH-strain 2 weeks, 1 month, 3 months or 4 months later, showed almost 100 per cent survival rates, while normal mice all died after 5–9 days.

Key words: Activated mouse macrophages; morphology; properties.

A. Reikvam, Institute of Physiology, University of Oslo, Karl Johansgt. 47, Oslo 1, Norway.

Received 14.x.74 Accepted 7.xi.74

It has been demonstrated by *Cohn & Benson* (6, 7) that normal mononuclear phagocytes are stimulated if cultured in a medium containing high concentrations of newborn calf serum (NBCS). In a previous paper (26) we have confirmed these results and in addition shown that *in vitro* activated macrophages have an increased capacity to phagocytose two intracellular micro-organisms, *Toxoplasma gondii* and *Listeria monocytogenes.* The capacity to restrict intracellular growth, however, was not significantly increased.

The non-specific *in vitro* activation of mononuclear phagocytes has obviously many morphological and biochemical properties in common with immunological macrophage activation in the intact animal; in fact, no

* Present address: Institute of Medical Biology, University of Tromsö, Tromsö.

** Present address: National Institute of Public Health, Oslo.

essential differences have been reported. It has therefore been speculated that they may represent the same kind of processes (8, 4). To our knowledge, a comparison of the phagocytic and microbicidal function of these two types of activated macrophages has not been undertaken. Such comparison has been the aim of the present investigation.

The mechanism by which mononuclear phagocytes kill micro-organisms is still poorly understood, although it is believed that lysosomes somehow may be involved (5, 11, 3). Furthermore, stimulation of lysosomes and lysosomal enzymes has been recognized as one of the most prominent features during *in vivo* activation (4) and indeed, also in non-specific *in vitro* activation of macrophages (6). Our interest was therefore focused on lysosomes and a quantitative evaluation of two lysosomal enzymes has been performed.

MATERIALS AND METHODS

Animals. Specific pathogen-free NMRI/Bom-mice, aged 1–3 months, weight 22–30 g, were employed. In one type of experiment, mice infected with the Beverly strain were later challenged with the RH-strain to ascertain whether they had become resistant against the latter strain.

Harvesting of mouse mononuclear phagocytes. Mouse peritoneal cells were obtained from unstimulated peritoneal cavities by lavage with medium 199 containing 20 per cent NBCS and antibiotics as described previously (26).

The cell suspension from each mouse was handled separately. After withdrawal of a sample for cell counts, the cells from each mouse were transferred to one or two Leighton tubes. Macrophages for biochemical analysis were cultured on the bottom of the Leighton tubes while macrophages destined for infection were cultured on "flying cover slips" within the tubes. After incubation for one hour the supernatant was discarded and the adherent cells washed twice with medium 199. Subsequently, medium 199 containing 50 per cent newborn calf serum (NBCS) and antibiotics was added and incubation continued in an atmosphere of 5 per cent CO_2 in air.

In vitro activation of macrophages. The normal peritoneal macrophages were activated by culture in a 50 per cent NBCS medium for 4 days, as described elsewhere (26). They were then used for the infection experiments.

In vivo activation of macrophages through infection. Peritoneal cells were harvested from the peritoneal cavities of mice 14 days after a subcutaneous injection containing 10–15 cysts of the Beverly strain of *T.gondii*. After processing as described above, the cells were taken for biochemical assay after the initial incubation for one hour, or used for *in vitro* infection studies 3 hours later.

Toxoplasma gondii

Beverly strain. Mice infected with the Beverly strain originally isolated by Beverly were kindly supplied by professor *Lycke*, Department of Microbiology, Sahlgrenska Hospital, Gothenburg, Sweden. This strain was originally considered to be low virulent, but during the period in which it had been kept in their laboratory, it had become more virulent. A dose which previously had been non-lethal killed now a certain proportion of the mice. The strain was passaged to new mice every third month. The brains of infected mice were then dissected out and homogenized with 0.9 per cent NaCl in a mortar. The number of cysts per drop were counted under the microscope and by dilution adjusted to 10–15 cysts/0.3 ml which was injected subcutaneously into each mouse. Usually 15–30 per cent of the mice died 1–2 weeks later. After 2 weeks, further deaths among the infected mice were few.

RH-strain. Details concerning processing of the RH-strain have been given elsewhere (26).

Listeria monocytogenes

A rough strain was prepared and used as described (26).

Infection of macrophage monolayers. Evaluation of phagocytosis and intracellular fate of micro-organisms. Each macrophage monolayer was infected with $2–3 \times 10^6$ *T. gondii* (RH-strain) or with 10^7 *L. monocytogenes*. Antibiotics were omitted from the growth medium used in the experiments with *L. monocytogenes* (26). The infection procedure has been described previously (26).

One of the duplicate tubes containing cells from one and the same mouse was harvested after the phagocytic step (1 hour), and the other at the end of the culture period (18 hours). Cover slips were stained with Giemsa; the number of macrophages per high magnification field (95 × objective, 20 fields counted), and the number of micro-organisms per macrophage (100–300 macrophages scored) were counted.

Serological assay. Mice infected with the Beverly strain were exsanguinated by heart puncture immediately after the peritoneal cells were washed out. The sera obtained were examined for toxoplasma antibodies, using the Dye Test (30).

Biochemical determination. Normal macrophages were cultured *in vitro* for 1, 2 and 4 days were

harvested for biochemical assay. *In vivo* activated macrophages were cultured on glass for one hour in order to have them separated from the non-adherent cells (lymphocytes). The cells were processed in the following manner: The media were removed and the monolayers washed four times with 0.9 per cent NaCl. Then, 2 ml of saline were added to each Leighton tube and the cells were wiped off the glass with a rubber policeman. This procedure was chosen because high concentrations of both trypsin and EDTA were unable to loosen the cells. Cell suspensions thus obtained were stored at − 20° C. In order to destroy the cells and activate the hydrolases, the cell suspensions were frozen and thawed 10 times on a methanol-dry ice bath.

Acid phosphatase. (E. C. 3. 1. 3. 2.) was assayed at pH 5.0 with β-glycerophosphate (Sigma) as substrate (1), using 0.25 ml aliquots of the suspension in a total incubation volume of 0.5 ml. After 4 hours at 37° C, incubation was terminated and liberated phosphate was determined by the method of *Fiske* & *Subbarow* (9).

β-glucuronidase. (E. C. 3. 2. 1. 31.) For the determination of this enzyme, 0.2 ml of the suspension was incubated in a total volume of 0.5 ml for 4 hours at 37° C with phenolphtalein glucuronic acid (Sigma) as substrate at pH 5.0 (12).

It applies to both enzymes that one unit of activity will hydrolyse one μmole of substrate in one min. Activity is expressed as milli-units/mg protein (specific activity) or as milli-units/Leighton tube (total activity).

Protein was measured by the method of *Lowry et al.* (19), using crystalline bovine serum albumin (Sigma) as a standard.

RESULTS

Activation of macrophages. Usually, 15–30 per cent of the mice infected with the Beverly

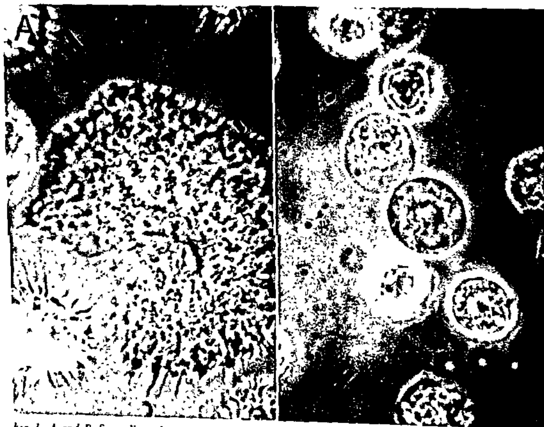


Fig 1, *A* and B. Spreading of macrophages on glass. Phase contrast, × 1460. A. Macrophages harvested from a mouse with a 14 days old Beverly strain infection, and cultured for 45 min *in vitro*. Spreading is already considerable. Numerous phase-dense granules surround the nucleus. B. Control. Normal macrophages cultured for 45 min under the same conditions.

Table 1. Phagocytic Capacities of Activated Macrophages

Activation	Extracellular protozoa per tube ($\times 10^6$)	Cell density (cell no. per microscop. field)
In vivo	1.40±0.14	20.6±3.3
In vitro	1.20±0.06	20.1±1.3

Number of protozoa left in the supernatant of *in vitro* and *in vivo* activated macrophage cultures after 1 hour of *T. gondii* infection. Cell densities on the cover slips are also given. Mean ± 1 standard error for 5 cultures is given.

strain of *T. gondii* died. Death occurred almost always between day 7 and day 14. The survivors had a disseminated infection when examined on day 14. The liver and the spleen were enlarged. Occasionally, calcium deposits were present in the peritoneum, whereas peritoneal exudate was not found. Sera from the animals used were all Dye Test positive, titres ranging from 1/16 to 1/128, 1/64 being the median titre. We considered macrophages from mice with a 14 days old Beverly infection to be highly activated (22,

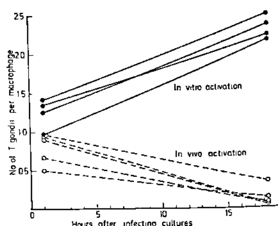


Fig. 2. Fate of *T. gondii* in macrophages. Macrophages cultured for 4 days in NBCS and macrophages just removed from mice with a 14 days old Beverly infection were infected with the RH-strain of *T. gondii* for 1 hour. Subsequently, half the cultures were harvested, the others were washed, re-incubated for 18 hours and then harvested. Number of *T. gondii* in 300 macrophages were counted.

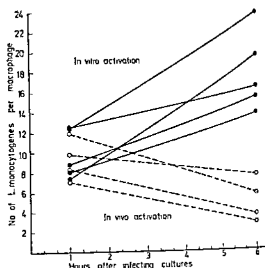


Fig. 3. Fate of *L. monocytogenes* in macrophages. Same groups of macrophages as in Fig. 2 were infected with *L. monocytogenes* for 1 hour. Subsequently, half the cultures were harvested, the cells of the others were washed, re-incubated and harvested after 6 hours. Number of *L. monocytogenes* in 150 macrophages scored.

31). They showed increased spreading ability on glass, were larger than normal macrophages, and contained typical perinuclear phase-dense granules (lysosomes) which were not observed in normal macrophages (Fig. 1A and B). The phase-dense granules seemed to be smaller than those present in *in vitro* activated cells, but otherwise the morphological appearance of the two types of activated cells was rather similar. Occasionally, protozoa were observed in *in vivo* activated cells proving definitively that the infection was generalized.

Phagocytosis and intracellular fate of RH T. gondii. The number of protozoa left in the supernatant fluid one hour after the two types of activated macrophage cultures had been infected with approximately 2.5×10^6 RH trophozoites was counted in a haemocytometer (Table 1). The number of protozoa left in the *in vitro* activated group was a little lower, but otherwise the numbers were of the same magnitude. The macrophage density was approximately the same in the two groups (Table 1).

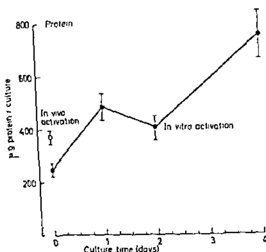


Fig. 4. Protein content of macrophages. Normal macrophages harvested after different times of *in vitro* culturing in 50 per cent NBCS. Macrophages from animals with a 14 days old Beverly infection cultured for 1 hour to separate them from non-adherent cells. Mean ± 1 standard error of 8 cultures is shown.

The number of protozoa per cell was 56 per cent higher in the *in vitro* activated cells than in the *in vivo* activated cells one hour after the start of infection, infected and non-infected cells included (Fig. 2). After 18 hours, the protozoa/cell ratio of *in vitro* activated macrophages was found to be markedly increased. In contrast, *in vivo* activated cells exhibited a fall in the protozoa/cell ratio during the interval between he 1-hour and the 18-hour determination and few protozoa were found. Characteristic osettes of protozoa, indicative of multiplication, were often observed in *in vitro* activated macrophages, but were not present in those activated *in vivo*.

The media used for washing the *in vivo* activated macrophages after one hour of incubation *in vitro* were collected and examined for Toxoplasma antibodies by the Dye Test. They were all negative.

Phagocytosis and intracellular fate of L. monocytogenes. Macrophage monolayers were infected with about 10⁷ *L. monocytogenes*/culture. Bacteria/cell ratios of *in vitro* and *in vivo* activated macrophages were nearly the same at the one-hour evaluation (Fig. 3), showing that the phagocytic capacities were approximately the same. At 5 hours, the ratio of the *in vitro* macrophages had increased, while that of the *in vivo* activated macrophages had decreased.

Protein content of activated macrophages. Culture of normal macrophages in a medium containing 50 per cent NBCS would increase the cell size and an increase in protein content was expected. From day 0 to day 4, a three-fold increase in protein content per Leighton tube was found (Fig. 4). Since the

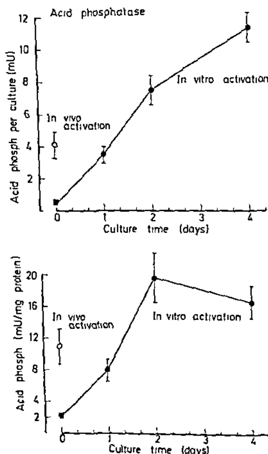


Fig. 5 (upper fig.) and *Fig. 6* (lower fig.). Acid phosphatase content of macrophages. Same type of cultures as in Fig. 4. The content of acid phosphatase per culture was measured and expressed in this way in Fig. 5, while enzyme content per mg protein is given in Fig. 6. Mean ± 1 standard error of 8 cultures is shown.

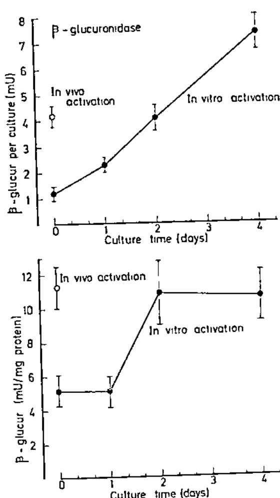


Fig. 7 (upper fig.) and *Fig. 8* (lower fig.). β-glucuronidase content of macrophages. Same macrophage cultures as in Figs. 4–6. Enzyme content per culture is given in Fig. 7 and per mg protein in Fig. 8.

number of cells per culture was approximately equal in the two situations, a three-fold increase in amount of protein per cell had taken place. *In vivo* activated macrophages also had higher protein content than normal macrophages (~ 1½ ×).

Acid phosphatase of activated macrophages. As regards *in vitro* activated macrophages, the activity per culture which reflects the activity per cell, increased throughout the culture period and was 21.5 times higher than that of unstimulated cells after 4 days. *In vivo* activated cells had an enzyme content which was increased 7.7 times as compared with that of unstimulated cells (Fig. 5). The corresponding increases per mg of protein were 6.9 × and 4.6 ×, respectively (Fig. 6).

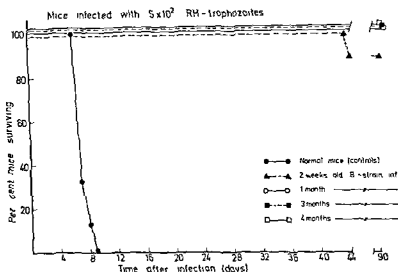
β-glucuronidase of activated macrophages. Activity per Leighton tube of *in vitro* activated cells increased 6.0 times during the 4-day-culture period (Fig. 7). By way of comparison, *in vivo* activated macrophages were found to have an enzyme content per tube which was 3.4 times over that of the controls. The corresponding values per mg protein were 2.0 × for *in vitro* activated macrophages and 2.2 × for the *in vivo* activated ones (Fig. 8).

Survival rates of Beverly strain infected mice after challenge with the RH strain. The survival rates after RH-challenge of normal mice and of mice with a Beverly infection persisting for 2 weeks, 1 month, 3 months and 4 months were recorded. When a challenge dose of 5×10^2 trophozoites was used (Fig. 9), the mean survival time of normal mice would be 8.1 ± 0.2 days and, using doses of 5×10^3 trophozoites (Fig. 10), it would be 6.0 ± 0.3 days. All groups of Beverly infected mice were remarkably resistant, 63–100 per cent of the mice surviving an observation period of 3 months.

DISCUSSION

The present experiments show that macrophages activated in two different ways, i.e. immunologically *in vivo* and non-specifically *in vitro*, were similar in morphological respects and that the lysosomal enzymes acid phosphatase and β-glucuronidase were raised in both types of cells; as regards most parameters, however, to the highest level in the *in vitro* activated macrophages. Despite this fact, the *in vivo* activated macrophages were much more effective in restricting intracellular growth of *T. gondii*, and also of *L. monocytogenes*, a micro-organism which is unrelated to the one used in the activation process.

In contrast to this, the phagocytic capacity was of the same magnitude in the two cell types. *In vivo* activated cells showed a num-



ig. 9. Survival rates of Beverly strain immunized mice. Normal mice (controls) and mice with a Beverly infection persisting for 2 weeks, 1 month, 3 months and 4 months were challenged with 5×10^2 RH-trophozoites. Each group of Beverly strain infected mice consisted of 9–10 mice. 15 normal mice were used.

ber of *T. gondii* per macrophage after the phagocytic step lower than that in the *in vitro* activated macrophages. This could indicate a lower phagocytic capacity of the former cells. Since the number of protozoa left extracellularly did not differ markedly, however, the interpretation must be that the *in vivo* activated cells had killed and disintegrated a large proportion of the trophozoites after one hour.

Cellular immunity is more important than humoral immunity for the suppression of

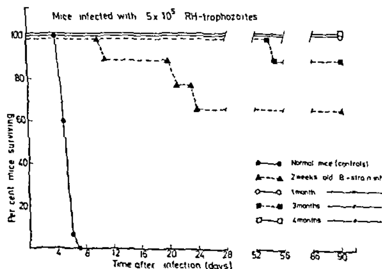


Fig. 10. Same groups and number of mice as in Fig. 9, but these mice were challenged with a 1000 times larger dose (5×10^5 RH-trophozoites).

intracellular micro-organisms. This applies equally well to *L. monocytogenes* as to *T. gondii* infections (10, 14, 21, 24). The ultimate killer cells in these infections are the activated macrophages (20, 23, 25, 34). A minor role of toxoplasma antibodies cannot be ruled out immediately in our experiments where *in vivo* activated macrophages were infected with the RH-strain. Such antibodies could not be detected in the growth medium, as judged by the Dye Test, but they may still have been present and bound to the macrophage membrane as cytophilic antibodies. Such antibodies probably do not bind to *L. monocytogenes.* Nevertheless, *in vivo* activated macrophages had a remarkable ability to kill this bacterium too, indicating that the process of *in vivo* activation *per se* was essential for the antibacterial efficiency of the cells.

The present experiments concerning survival rates showed that the surviving mice infected with the Beverly-strain were highly capable of suppressing a RH-infection and not only the isolated macrophages. The RH-strain is extremely pathogenic for mice, the LD_{100} probably being less than 10 protozoa (28, 29). The mice with a 2 weeks old Beverly infection had a higher death rate than the others, but this did not necessarily mean that maximal macrophage activation had not been achieved at this time. The higher death rate might be due to a poor general condition of these animals after the severe and almost lethal primary infection. The protection was still fully operative even 4 months after the Beverly infection. A long-lasting protection after immunization with *T. gondii* (27) has also been reported by others; it is probably due to cysts which remain in the tissues and thereby exert a continual immunological stimulus (27).

Lysosomes are engaged in the breakdown of organic materials, and are also assumed to play a key role in the killing of the intracellular micro-organisms. The clearly demonstrated fusion between the phagocytic vacuole and lysosomes lends even more support to this view. Hence, it is interesting that our *in vitro* activated cells as well as the *in vivo* activated cells were heavily endowed with lysosomes, but the killing ability of the former cells was definitely inferior to that of the latter. There are three possible explanations of these observations: i) The lysosomes induced during *in vitro* culture may somehow be defective. ii) The mechanism initiating fusion between the phagocytic vacuole and lysosomes must be selectively stimulated during macrophage activation; this did not occur during *in vitro* activation. iii) Fusion between the phagocytic vacuoles and lysosomes does not occur until the micro-organisms are recognized as dead. The last notion implies that an important component of micro-organism inactivation must be localized outside the lysosomes and that the lysosomes are scavengers only, responsible for disintegration of micro-organisms that are already dead.

None of the three hypotheses can be immediately rejected. To our knowledge, however, the literature does not provide evidence in support of hypothesis (i) or (ii), while there is some experimental support for (iii). *Armstrong et al.* favour this hypothesis on the basis of their experiments with living and dead mycobacteria (2). They also showed that *Mycobacterium lepramurium* was able to proliferate within the phagolysosome (13). Furthermore, phagocytic vacuoles containing live *T. gondii* differed markedly from those with dead protozoa (15). *Kochan & Golden* (17, 18) recently reported that non-esterified fatty acid from disrupted macrophages could account for the antibacterial effect of the cell lysated. These observations might also indicate that non-lysosomal structures are of importance for the killing process in macrophages. It should also be noted that the killing mechanisms claimed to be operative in granulocytes have not been shown to be important in macrophages (3, 16, 32, 33). Our two types of activated macrophages with quite different microbicidal capacities might provide a favourable basis for investigations into such mechanisms in macrophages.

REFERENCES

1. *Appelmans, F., Wattiaux, R. & DeDuve, C.:* Tissue fractionating studies. 5. The association of acid phosphatase with a special class of cytoplasmic granules in rat liver. Biochem. J. *59:* 438–445, 1955.
2. *Armstrong, J. A. & Hart, P. D'Arcy:* Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes with phagosomes. J. exp. Med. *134:* 713–740, 1971.
3. *Axline, S. G.:* Functional biochemistry of the macrophage. Seminars Hemat. *7:* 142–160, 1970.
4. *Blanden, R. V.:* Modification of macrophage function. J. reticuloendoth. Soc. *5:* 179–202, 1968.
5. *Cline, M. J.:* Bactericidal activity of human macrophages: analysis of factors influencing the killing of *Listeria monocytogenes*. Infect. Immun. *2:* 156–161, 1970.
6. *Cohn, Z. A. & Benson, B.:* The differentiation of mononuclear phagocytes. Morphology, cytochemistry and biochemistry. J. exp. Med. *121:* 153–169, 1965 a.
7. *Cohn, Z. A. & Benson, B.:* The in vitro differentiation of mononuclear phagocytes. II. The influence of serum on granule formation, hydrolase production, and pinocytosis. J. exp. Med. *121*, 835–848, 1965 b.
8. *Cohn, Z A., Hirsch, J. G. & Fedorko, M.:* The in vitro differentiation of mononuclear phagocytes. IV. The ultrastructure of macrophage differentiation in the peritoneal cavity and in culture. J. exp Med *123:* 747–755, 1966.
9. *Fiske, C. H. & Subbarow, Y.:* The colorimetric determination of phosphorus. J. biol. Chem *66:* 375–400, 1925
10. *Frenkel, J K.:* Adoptive immunity to intracellular infection. J. Immunol. *98:* 1309–1319, 1967.
11. *Furth, R. van, Hirsch, J. G. & Fedorko, M. E.:* Morphology and peroxidase cytochemistry of mouse promonocytes, monocytes and macrophages. J. exp. Med. *132:* 794–805, 1970.
12. *Gianetto, R. & DeDuve, C.*, Tissue fractionating studies 4. Comparative study of the binding of acid phosphatase, β-glucuronidase and cathepsin by rat-liver particles. Biochem. J *59:* 433–438, 1955.
13. *Hart, P. D'Arcy, Armstrong, J A., Brown, C. A & Draper, P.:* Ultrastructural study of the behaviour of macrophages towards parasitic mycobacteria. Infect. Immun. *5:* 803–807, 1972.
14. *Hoff, R. L. & Frenkel, J. K* · Cell mediated immunity against Besnoitia and Toxoplasma in specifically and cross-immunized hamsters and in cultures. J. exp. Med. *139:* 560–580, 1974.
15. *Jones, T. C. & Hirsch, J. G.:* The interaction between *Toxoplasma gondii* and mammalian cells. II. The absence of lysosomal fusion with phagocytic vacuoles containing living parasites. J. exp. Med. *136:* 1173–1194, 1972.
16. *Klebanoff, S. J.:* Iodination of bacteria: A bactericidal mechanism. J. exp. Med. *126:* 1063–1078, 1967.
17. *Kochan, I. & Golden, C. A.:* Antimycobacterial effect of lysates prepared from immunologically activated macrophages. Infect. Immun. *8:* 389–394, 1973.
18. *Kochan, I. & Golden, C. A.:* Immunological nature of antimycobacterial phenomenon in macrophages. Infect. Immun. *9:* 249–254, 1974.
19. *Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J.:* Protein measurement with the folin phenol reagent. J. biol. Chem. *193:* 265–275, 1951.
20. *Mackaness, G. B.:* Cellular resistance to infection. J. exp. Med. *116:* 381–406, 1962
21. *Mackaness, G. B.:* The influence of immunologically committed lymphoid cells on macrophage activity in vivo. J. exp. Med *129:* 973–992, 1969.
22. *Mackaness, G. B.:* Cellular immunity. In: Furth, R. van (Ed.): Mononuclear phagocytes. Blackwell Scientific Publications, Oxford 1970, pp. 461–476.
23. *McGregor, D. D. & Koster, F. T.:* The mediator of cellular immunity. IV. Cooperation between lymphocytes and mononuclear phagocytes. Cell. Immunol. *2:* 317–325, 1971.
24. *Miki, K. & Mackaness, G. B.:* The passive transfer of resistance to *Listeria monocytogenes*. J. exp. Med. *120:* 93–103, 1964.
25. *Nathan, C. F., Karnovsky, M. L. & David, J. R.:* Alterations of macrophage functions by mediators from lymphocytes. J. exp. Med. *133:* 1356–1376, 1971.
26. *Reikvam, A. & Hoiby, E. A.:* Phagocytosis and microbicidal capacity of mouse macrophages non-specifically activated *in vitro*. Acta path. microbiol. scand. Sect. C, *83:* 121–128, 1975.
27. *Remington, J. S. & Merigan, T.:* Resistance to virus challenge in mice infected with protozoa or bacteria. Proc. Soc. exp. Biol. (N.Y.) *131:* 1184–1188, 1969.
28. *Ruskin, J., McIntosh, J. & Remington, J. S.:* Studies on the mechanisms of resistance to phylogenetically diverse intracellular organisms. J. Immunol. *103:* 252–259, 1969.
29. *Ruskin, J. & Remington, J. S.:* Resistance to intracellular infection in mice immunized with toxoplasma vaccine and adjuvant. J. reticuloendoth. Soc. *9:* 465–479, 1971.
30. *Sabin, A. B. & Feldman, H. A.:* Dyes as

microchemical indicators of a new immunity phenomenon affecting a protozooan parasite (Toxoplasma). Science *108:* 660–663, 1948.

31. *Saito, K.* & *Suter, E.:* Lysosomal acid hydrolases in mice infected with BCG. J. exp. Med. *121:* 727–739, 1965.

32. *Selvara, R. J.* & *Sbarra, A. J.:* Relationship of glycolytic and oxidative metabolism to particle entry and destruction in phagocytosing cells. Nature (London) *211:* 1272–1276, 1966.

33. *Simmons, S. R.* & *Karnovsky, M. L.:* Iodinating ability of various leukocytes and their bactericidal activity. J. exp. Med. *138:* 44–63, 1973.

34. *Simon, H. B.* & *Sheagren, J. N.:* Cellular immunity in vitro. I. Immunologically mediated enhancement of macrophage bactericidal capacity. J. exp. Med. *133:* 1377–1389, 1971.

Acta path. microbiol. scand. Sect. C, 83: 139–143, 1975

INTERACTION OF CONCANAVALIN A WITH HUMAN NEUTROPHIL GRANULOCYTE FUNCTION *IN VITRO*

Christian Koch

The University Clinic for Infectious Diseases, and Statens Seruminstitut, Department of Clinical Microbiology, Blegdamshospitalet, Copenhagen, Denmark

Koch, C. Interaction of concanavalin A with human neutrophil granulocyte function *in vitro*. Acta path. microbiol. scand. Sect. C, 83: 139–143, 1975.

The ingestion of *Staphylococcus aureus* by human neutrophil granulocytes was inhibited by the addition of concanavalin A (Con A). Intracellular killing of the bacteria and reduction of nitroblue-tetrazolium (NBT) was enhanced, however, indicating stimulation of oxydative metabolism by Con A. This stimulation may in part be due to agglutination of the cells, since NBT reduction was less prominent when agglutination was prevented. The effects of Con A were different from those of metabolic inhibitors of glycolysis, and the decreased ingestion is probably caused by specific binding of Con A to cell membrane glycoproteins leading to derangement in normal membrane function.

Key words: Neutrophil granulocyte function; concanavalin A.

Chr. Koch, M.D., Blegdamshospitalet, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark.

Received 24.ix.74 Accepted 9.x.74

Concanavalin A (Con A) binds to—and agglutinates erythrocytes and polymorphonuclear leucocytes and has been shown to inhibit phagocytosis by neutrophil granulocytes (2). This inhibition is specific, in the sense that particle-uptake is decreased while another membrane function, the transport of adenine and adenosine is unimpaired (2). Con A also binds specifically to both T- and B-lymphocytes and, in the case of T-lymphocytes, acts as a mitogen, indicating stimulation of cellular metabolism (15).

The present experiments were designed to analyse the effects of Con A upon ingestion as well as subsequent intraleucocytic killing of viable bacteria by human neutrophil granulocytes. In addition, the effect of Con A upon the oxydative metabolism of these cells was evaluated indirectly by recording intracellular reduction of the electron-receptor, nitroblue-tetrazolium (NBT) (1).

MATERIALS AND METHODS

Ingestion and killing of *Staphylococcus aureus*, 502A, were determined as described previously (9). Briefly, 2.5×10^6 polymorphonuclear leucocytes were incubated with an approximately equal number of colony-forming units (CFU) bacteria in the presence of 10 per cent serum. Total, and intracellular CFU, and intracellular CFU in cells, treated with 10 mM sodium azide (NaN_3), were determined after 2 and 4 hours' incubation. NaN_3 at this molarity effectively blocks intracellular killing without interfering with ingestion (10). Con A (Pharmacia Fine Chemicals, Uppsala, Sweden, batch no. 4000), freshly dissolved in 0.154 M saline, was added in equal volumes at the start of incubation to give final concentrations of 0, 50, and 250 µg per ml The influence of Con A upon

ingestion was expressed as the number of CFU in NaN_3-treated cells *exposed to Con A, in per* cent of corresponding values in the absence of Con A. The influence upon *killing* was expressed as the ratio: number of CFU in NaN_3-treated over *untreated cells exposed to Con A, in per cent of* corresponding values in the absence of Con A (Fig. 1).

Additional studies were done *to compare the effects of Con A with those of metabolic inhibitors* of glycolysis. Sodium fluoride (Merck, Darmstadt, *Germany*), *at a final concentration of 20* mM, or iodo-acetic acid (Merck), at 0.1 mM, *were added at the start of incubation. Since NaN_3* was not employed for these studies, the results are *expressed as total, and intracellular CFU in the* presence of additive (Con A, or inhibitor) over *simultaneously obtained control* values in the absence of additive (Table 1).

NBT studies. Leucocytes were washed and resuspended in gelatinized, heparinized Hank's balanced salt solution containing 4.7 per cent human serum albumin (Cutter Lab. U.S.A., lot no. K 9777), *adjusted to pH 7.4 with 2.8 per cent* sodium bicarbonate. 0.1 ml of this suspension was mixed with an equal volume of NBT solution, and NBT-test carried out as described previously (11). *Studies were done with final concentrations of* 2.5×10^6, and 2.5×10^5 polymorphonuclear leucocytes per ml; *the high density thus correspond*ing to the density employed for functional studies. *Con A was added at the start of incubation to* give final concentrations as in functional studies. *These experiments were also repeated with substi*tution of 10 per cent fresh serum for albumin in *the medium.*

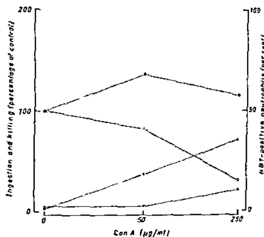


Fig. 1. Neutrophil granulocytes were incubated with *Staph. aureus* in medium containing 10 per cent human serum; density of neutrophils: 2.5×10^6/ml. Con A was added at start of incubation at concentrations of 50 and 250 µg/ml. The number of colony-forming units (CFU) bacteria beeing ingested (○——○), and the number of ingested CFU beeing killed (●——●) were determined after 4 hours' incubation , as described under methods, and expressed as these values in the presence of Con A, in per cent of corresponding values in the absence of Con A. Mean of three experiments. For NBT studies, leucocytes were suspended in medium containing 4.7 per cent human albumin and exposed to NBT in the absence and presence of Con A at the same concentrations. Studies were done with densities of neutrophils of 2.5×10^6 (▲——▲) and 2.5×10^5 (△——△) per ml. 250 neutrophils were evaluated and the percentage of NBT reducing cells recorded. Mean of three experiments.

RESULTS

Fig. 1 shows graphically the results of Con A studies expressed as mean values of three separate experiments. Addition of Con A caused a decrease in the ingestion of *Staph. aureus* to 83 per cent of control values at 50 µg Con A per ml, and 32 per cent at 250 µg/ml Intraleucocytic killing of the bacteria that were ingested was unimpaired, however, or in fact slightly increased to 137 *and 117 per cent of control* values at 50, and 250 µg Con A per ml.

Table 1 shows that the actions of Con A were different from those of metabolic inhibitors of glycolysis. Con A caused an increase *in total* CFU which was slight at 50 µg/ml, but pronounced at 250 µg/ml. Intracellular CFU, however, were decreased with increasing Con A concentration. Fluoride, and to a lesser degree iodo-acetate, also increased total *CFU, but contrary to* Con A, intracellular CFU were increased by both agents. In the present system, an increase in intracellular CFU reflects defective killing, while an increase in total CFU reflects defective inges*tion* (9). *Accordingly,* Con A, especially at 250 µg/ml, and fluoride, but not iodo-acetate, caused inhibition of *ingestion.* Both fluoride and iodo-acetate caused inhibition of intracellular killing which was normal or increased by Con A.

Fig. 1 also shows that the addition of

TABLE 1. *Effects of Con A and Metabolic Inhibitors of Glycolysis upon Ingestion and Intraleucocytic Killing of Staphylococcus aureus by Human Neutrophil Granulocytes*

Agent	Total CFU Ratio: With agent / Without agent mean	range	Intracellular CFU Ratio: With agent / Without agent mean	range
Sodium fluoride, 20 mM*	6.34	(3.55– 9.12)	7.87	(7.10–8.63)
Iodoacetic acid, 0.1 mM*	2.51	(2.06– 2.96)	4 83	(3.24–6.41)
Con A, 50 μg/ml†	3.86	(1.40– 8.77)	0.76	(0.64–0.97)
Con A, 250 μg/ml†	41.6	(17.1 –90.1)	0.31	(0.27–0.36)

Neutrophil granulocytes were incubated with *Staph. aureus in vitro*. Total and intracellular colony-forming units (CFU) bacteria were determined after 4 hours' incubation. The data are the ratios of these values in the presence of added agent over simultaneously obtained values in the absence of agent.

* two experiments.

† three experiments.

Con A results in an increase in the number of neutrophils that actively reduce NBT. This effect was clearly demonstrable when the density of the cell population used was 2.5×10^6 per ml, mean values being 1.6, 18.3, and 35.5 per cent at 0, 50, and 250 μg Con A per ml. This effect was, however, clearly diminished when the density of the cell population was reduced ten-fold to 2.5×10^5, where Con A at 50 μg/ml caused no increase and 250 μg/ml only a moderate increase from 2.4 to 10.2 per cent NBT reducing cells.

Inspection of the smears also revealed agglutination of neutrophils, which was clearly more pronounced at the high density of cells. Thus, Con A at 50 μg/ml caused moderate agglutination at 2.5×10^6, but no agglutination at 2.5×10^5 neutrophils per ml. 250 μg Con A caused marked agglutination at the high density and moderate at the low density of cells. In the absence of Con A the cells were nearly always located singly.

Repeating the studies, but substituting fresh serum for albumin, did not change the influence of Con A upon agglutination or NBT reduction. There was rather a tendency for neutrophils in greater number to reduce NBT in the presence of Con A (data not shown).

DISCUSSION

These experiments demonstrate decreased ingestion, but slightly enhanced killing of *Staph. aureus*, and increased NBT reduction by human neutrophils in the presence of Con A. Only the decreased ingestion could theoretically be explained by inactivation by Con A of serum-opsonins (4), and fresh serum did not diminish NBT reduction in the presence of Con A; therefore the neutrophil changes are probably caused by specific reversible binding of Con A to cell membrane glycoproteins, as demonstrated previously (2). The effects of Con A are clearly different from those of fluoride and iodo-acetate which caused inhibition of killing, as would be expected, since these agents inhibit oxydative metabolism (3, 8). Fluoride caused some inhibition of ingestion, which can be explained by the effect of this agent upon the level of cellular cyclic adenosine monophosphate (5, 6).

Con A may bind to, or mask, specific receptors for IgG or C3 (12, 13), but uptake of inert particles in the absence of serum is also inhibited by Con A (2), wherefore it appears more likely that inhibition of uptake is caused by structural changes in the cell surface or membrane that interfere with nor-

mal membrane-internalization. Since at the pH employed, 7.4, Con A is present in polymers, a more rigid cell membrane could be caused by cross-linking of adjacent Con A receptor sites or Con A might interfere with a hypothetical fusion of receptor sites, necessary for normal ingestion. Con A also inhibits the patch and cap formation induced in lymphocytes by anti-immunoglobulin, which has been suggested to result from changes in or on the lymphocyte membrane that inhibit the free diffusion of immunoglobulin receptors (16).

Con A, besides inhibiting ingestion, also caused increased NBT reduction and slightly enhanced killing, which both signify stimulation of oxydative metabolism (1, 8). Binding of Con A to guinea-pig polymorphonuclear cells causes reversible stimulation of oxydative metabolism which cannot be explained by ingestion of Con A-aggregates (14). The present findings indicate a similar effect of Con A upon human neutrophils, but although positive NBT staining was observed in singly located cells, the majority of NBT reducing cells were those that were agglutinated, and NBT reduction was clearly reduced when agglutination was in part prevented. This suggests that NBT reduction either was secondary to membrane to membrane interaction between individual cells, or augmented by such interaction. This is not quite unlikely since the mere adherence of neutrophils to glass surfaces may also induce NBT reduction (7).

These studies raise the question whether Con A is capable of direct and specific stimulation of metabolism in human neutrophils; this could possibly be solved in a system which effectively prevents adherence and agglutination. This question might be worth studying in view of the unclear mechanism of mitogenic action of Con A upon T-lymphocytes, particularly since Con A interferes with normal membrane function of both neutrophils and lymphocytes and in both cells stimulates metabolism, expressed in the neutrophil as enhanced oxydative metabolism, and in the lymphocyte as blastic transformation and mitosis.

This work was supported by grants from *The Michaelsen Foundation*, *The Danish Medical Research Council*, grants no. 512-591 -1289 -1940 -3120, and *The Foundation for the Advancement of Medical Science*, Copenhagen, Denmark. Mrs. *Ulla Hoiby* is thanked for excellent technical assistance.

REFERENCES

1. *Baehner, R. L. & Nathan, D. G.:* Quantitative nitroblue tetrazolium test in chronic granulomatous disease. New Engl. J. Med. *278*. 971–976, 1968.
2. *Berlin, R. D.:* Effect of concanavalin A on phagocytosis. Nature New Biology *235:* 44–45, 1972.
3. *Bodel, P. & Malawista, S. E.:* Phagocytosis by human blood leucocytes during suppression of glycolysis. Exp. Cell. Res. *56:* 15–23, 1969
4. *Bøg-Hansen, T. C.:* Crossed immuno-affinoelectrophoresis; an analytical method to predict the results of affinity chromatography. Analyt. Biochem. *56:* 480–488, 1973.
5. *Constantopoulos, A. & Najjar, V. A.:* Adenyl cyclase of polymorphonuclear leucocytes. Nature New Biology *243*. 268–269, 1973.
6. *Cox, J. P. & Karnovsky, M. L.:* The depression of phagocytosis by exogenous nucleotides, prostaglandins, and theophylline. J. Cell Biol *59:* 480–488, 1973.
7. *Gifford, R. H. & Malawista, S. E.:* A simple rapid micromethod for detecting chronic granulomatous disease of childhood. J. Lab. Clin. Med. 75· 511–519, 1970.
8. *Holmes, B., Page, A. R. & Good, R A* Studies of the metabolic activity of leucocytes from patients with a genetic abnormality of phagocytic function. J. Clin Invest. *46* 1422-1432, 1967.
9. *Koch, C.:* Neutrophil granulocyte function in vitro. Evaluation of a fluid-phase leucocyte-bacteria reaction system Acta path. microbiol. scand. Sect. B, *82:* 127–135, 1974.
10. *Koch, C.:* Effect of sodium azide upon normal and pathological granulocyte function. Acta path. microbiol. scand. Sect. B, *82:* 136–142, 1974.
11. *Koch, C.:* Studies on the nitroblue-tetrazolium staining induced in human neutrophils by bacterial products. Acta path microbiol scand. Sect. B, *81:* 266–268, 1973.
12. *Lay, W. H. & Nussenzweig, V.* Receptors for complement on leucocytes. J. Exp Med. *128*. 991–1007, 1968.

13. *Quie, P. G., Messner, R. P. & Williams, R. C., Jr.:* Phagocytosis in subacute bacterial endocarditis. Localization of the primary opsonic site to the Fc fragment. J. Exp. Med. *128:* 553–570, 1968.
14. *Romeo, D., Zabucchi, G. & Rossi, F.:* Reversible metabolic stimulation of polymorphonuclear leucocytes and macrophages by concanavalin A. Nature New Biology *243:* 111–112, 1973.
15. *Stobo, J. D., Rosenthal, A. S. & Paul, W. E.:* Functional heterogeneity of murine lymphoid cells I. Responsiveness to and binding of concanavalin A and phytohemagglutinin. J. Immunol. *108:* 1–17, 1972.
16. *Yahara, I. & Edelman, G. M.:* Restriction of the mobility of lymphocyte immunoglobulin receptors by concanavalin A. Proc. Nat. Acad. Sci. USA *69:* 608–612, 1972.

Acta path. microbiol. scand. Sect. C, 83: 144–156, 1975

NITROBLUE-TETRAZOLIUM (NBT)-REDUCTION BY HUMAN PERIPHERAL NEUTROPHIL GRANULOCYTES IN THE PRESENCE OF BACTERIAL ANTIGENS

Evidence for Immune Complex Ingestion

CHRISTIAN KOCH, NIELS HOIBY and ALLAN WIIK

The University Clinic for Infectious Diseases, and Statens Seruminstitut, Department of Clinical Microbiology, Blegdamshospitalet, Copenhagen. The Department of Pediatrics TG, with the Department of Pediatrics of Dronning Louises Børnehospital, Rigshospitalet, University of Copenhagen, Denmark

Koch, C., Høiby, N. & Wiik, A. Nitroblue-tetrazolium (NBT)-reduction by human peripheral neutrophil granulocytes in the presence of bacterial antigens. Evidence for immune complex ingestion. Acta path. microbiol. scand. Sect. C, 83: 144–156, 1975.

The NBT-reduction induced in human peripheral neutrophils by stimulation with *Pseudomonas aeruginosa* antigens was found to depend upon the presence of serum factors. The response of the cells to stimulation was enhanced using immune serum, containing multiple precipitating antibodies against these antigens. The response was diminished by heat-inactivation of serum, prior to reaction with the antigens, and by elimination of precipitating immune complexes. Direct immunofluorescence studies demonstrated ingestion by the neutrophils of specific antigens with participation of immunoglobulins G, M, A, and complement C3. This was found, using immune serum, but also using normal serum, containing low-titred, cross-reactive antibodies to *Ps. aeruginosa*. Similar results were also obtained, using antigens from other bacterial species. It is suggested that bacterial antigens, upon interaction with immune as well as with normal serum, form large immune complexes, that are easily ingested by neutrophils, and that immune complex ingestion is closely linked to stimulated NBT-reduction. These studies suggest an important role of normally occurring, cross-reactive antibodies to bacterial antigens in promoting rapid clearing of toxic material, or material otherwise damaging to the host.

Key words: Neutrophil granulocytes; immune complex ingestion.

Chr. Koch, Blegdamshospitalet, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark

eived 2.xi.74 Accepted 2.xi 74

Neutrophils from patients with bacterial infection have been shown to reduce nitroblue-tetrazolium (NBT) *in vitro* (17, 20) and this finding has been applied to a widely used diagnostic test in febrile disorders. The value of the NBT-test has, however, been discredited on the basis of varying results (24, 27) which may partly be caused by technical factors (4), but much confusion stems from the unresolved question of how the cells, during infection, are triggered in a manner which causes increased NBT-reduction *in vitro*.

NBT-reduction can be induced *in vitro* by endotoxin (18, 21), by filtrates of bacterial cultures (14, 17), and by soluble, antigenic constituents from sonicated bacteria (15) which seems, in all situations, to be mediated through reaction of the stimulant with specific antibodies with participation of complement (14, 15, 18). It was therefore an important finding when it was shown that precipitating bovine serum albumin (BSA)/anti-BSA complexes were highly active in inducing NBT-reduction (19).

The aim of the present investigations was to study more closely the mechanism of stimulation of the neutrophils with bacterial products. It was shown that the NBT-reduction, induced by complex systems of microbial constituents with multiple antigenic determinants, was mediated through the formation of large-size antigen/antibody complexes, using both normal and immune serum. The application of immunofluorescence studies furthermore provided evidence for active ingestion of antigen bound to several classes of immunoglobulin with participation of complement C3, in the presence of immune as well as normal serum. The same kinetics seemed to determine NBT-reduction as well as immune complex ingestion.

MATERIALS AND METHODS

Microorganisms and microbial antigens. Pseudomonas aeruginosa were cultured and harvested as described earlier (10). After washing in phosphate-buffered saline (0.154 M) (PBS), they were used live, or killed by heating (100° C/60 min) or treatment with 3 per cent formalin for 30 minutes, followed by washing in PBS. The *Ps. aeruginosa* standard-antigen (St-Ag) was obtained by sonication of bacteria from 4 different O-groups, and has been described previously (10). Antigens from *Escherichia coli, Streptococcus pyogenes, Diplococcus pneumoniae, Neisseria meningitidis,* and *Staphylococcus aureus* without protein A were prepared in a similar manner (12). St-Ag was insolubilized by treatment with glutaraldehyde according to *Avrameas & Ternynck* (1969).

Latex particles. Uncoated particles (0.81 μm) were obtained from Difco Lab., Detroit, USA. Particles were coated with St-Ag by incubation of 500 μl of the stock solution of particles with 500 μl St-Ag and 2 ml glycine-saline diluent (Hyland, Belgium) at 4° C for 72 hours, followed by washing and resuspension in 500 μl PBS. Particles coated with human gammaglobulin (RA-test, Latex globulin reagent) were obtained from Hyland, Belgium.

Antisera. Unconjugated, and fluorescein-isothiocyanate (FITC)-conjugated rabbit IgG, specific for human IgG, IgM, IgA, complement C3 (β_{1C}-globulin), and fibrinogen were obtained from Dakopatts, Copenhagen. The specificities and working titres of the conjugates were determined as described earlier (29). The fluorescein/protein (F/P) ratio, as estimated by optical density at 495 and 278 nm, was around 0.65 in all these conjugates. The *Ps. aeruginosa* standard-antiserum (St-Ab) was a pooled, concentrated rabbit antiserum against St-Ag, characterized previously (10). St-Ab was conjugated with FITC and fractionated by DEAE-sephadex chromatography by the method of *The & Feltkamp* (1970). The protein concentration was 5.6 mg per ml, with a F/P ratio of 0.40. That the FITC-St-Ab had retained specific antibody activity was secured by *crossed immunoelectrophoresis* (c.i.) with St-Ag against FITC-St-Ab, as described previously (11), and by blocking and absorption studies, as mentioned under results. Specificity in the present system was further secured by the studies given in Table 1.

Antigen/antibody complexes. Human serum albumin (HSA) and rabbit anti-HSA were kindly donated by Dr. *J. M. Rhodes,* Statens Seruminstitut, Copenhagen, and complexes formed as described by *Rhodes* (1964) with slight modifications. Precipitating complexes were formed by incubation of 2 ml antiserum with 1 mg HSA, corresponding to the point of equivalence as determined by immunodiffusion according to *Sewell* (1967). Incubations were done at 35° C for 1 hour, followed by 4° C for 48 hours. The precipitates were washed three times in 0.154 M saline by centrifugation at 48,200 × *g* for 30 minutes, and then suspended in 2 ml saline and kept at −20° C. Complexes, formed at 8 times antigen-excess, were made by addition of 8 mg HSA to one of the precipitates, followed by incubation for 72 hours at 4° C on a tilting table. After centrifugation (48,200 × *g*/30 min), the supernatant was removed and stored at −20° C.

Sera. Fresh frozen (−80° C) *immune* sera with multiple precipitating antibodies against St-Ag (11) were obtained from 6 children with cystic fibrosis (C.F.) and 3 adults with chronic bronchitis (Chr. Br.). *Normal* sera were similarly obtained from healthy adults, or from a pool from more than 20 healthy blood donors.

NBT Studies

A. Effect of St-Ag upon NBT-reduction by neutrophils in the presence and absence of serum

TABLE 1. *Specificity of FITC-St-Ab*

Reaction system	FITC-conjugate	Intra-cytoplasmic fluorescence (– to + + +)	Comments
Latex + serum + leucocytes	St-Ab	–	
St-Ag-coated Latex + serum + leucocytes	„	+ + +	Fine, granular
IgG-coated Latex + serum + leucocytes	„	–	
„	anti-IgG	+ + +	Fine, granular
Heat-killed *Ps. aeruginosa* + leucocytes	St-Ab	+ + +	10 % neutrophils contain 1–2 organisms with "rim"-like staining
„ + serum	„	+ + +	„ – but in nearly all neutrophils
Formalin-killed *Ps. aeruginosa* + leucocytes	„	+ + +	Nearly all neutrophils contain *diffusely stained organisms*
„ + serum	„	+ + +	„
Live *Ps. aeruginosa* + serum + leucocytes	„	+ + +	All neutrophils contain strongly stained organisms

factors. Buffy-coat leucocytes from normal adults were obtained by dextran-sedimentation, and washed three times and resuspended in gelatinized, heparinized Hank's balanced salt solution (gel-Hank) (15) containing 10 per cent fresh autologous serum, 10 per cent fresh autologous plasma, or 5 per cent HSA (Cutter Lab. USA) adjusted to pH 7.4 with 2.8 per cent $NaHCO_3$. Different dilutions of St-Ag in 0.154 M saline or undiluted St-Ag (as indicated in Fig 1) were added in 5 µl volumes to 0.1 ml volumes of the different cell suspensions or heparinized whole blood, and NBT-tests carried out as descibed earlier (14). After smearing and counterstaining, 250 consecutive neutrophils were evaluated and the percentage containing clearly identifiable formazan crystals recorded.

B. Effect of St-Ag in combination with fresh and heat-inactivated serum, and effect of removal of large immune complexes. Fresh and heat-inactivated (56° C/30 min) normal and immune sera were incubated with 1/10 volumes of dilutions of St-Ag (as indicated in Fig. 2) at 35° C for 10 minutes. 0.2 ml of each mixture was stored at 4° C, while the remainder was centrifuged at 48,200 × *g* for 30 minutes at 4° C, followed by removal of 0.2 ml of the supernatant Each of the removed portions was mixed with blood cells from 0.3 ml heparinized normal blood, washed and suspended in gel-Hank adjusted to pH 7.4, and NBT-tests carried out.

C. Effect of absorption of antibodies to St-Ag. 300 µl undiluted St-Ag was incubated with 6.0 ml fresh or inactivated serum, as outlined above. 0.6 ml was stored at 4° C, while the remainder was centrifuged (48,200 × *g*/30 min), the supernatant carefully removed, and 0.6 ml of this stored 300 µl St-Ag was again added to the remaining approximately 5.1 ml supernatant, and the procedure repeated; 4 times in all. The fractions obtained after each incubation were designated I to IV, and the supernatants designated Is to IVs. 0.2 ml of each fraction was used for NBT-tests, as outlined under *B*, and the rest used for counter-immunoelectrophoresis studies.

Counterimmunoelectrophoresis This was done to investigate the immune precipitin reactions in fractions I to IV and Is to IVs, obtained as outlined under *C*, according to the principles of Duquesnoy (1973). 10 × 10 cm glass plates were coated with a mixture of 0 8 per cent agarose (Indubiose A 37, L'industrie Biologique Française, batch no. FF 7231) and 0.2 per cent Special Agar-Noble (Difco, Detroit, USA) in barbital buffer, pH 8 6, ionic strength 0.02, thickness of gel 1.5 mm. Under these conditions, bacterial proteins migrate towards the anode, while rabbit and human IgG migrate towards the cathode (Høiby, N.:

unpublished observation). Wells were punched in 2 parallel rows, 3 mm apart. Samples of 10 μl St-Ag, diluted 1:5 in barbital buffer, were placed in the wells on the cathodic side of the plate; 10 μl of fractions I to IV and Is to IVs were added to the opposite (anodic) wells. On a second plate, the fractions were placed in the wells on the cathodic side and St-Ab was added to the opposite (anodic) wells. According to *Duquesnoy* (1973), a precipitin band will develop in the agar-agarose between the wells on the first plate if the fractions have an excess of antibody. If the fractions have an excess of antigen, a precipitin band will develop between the wells on the second plate. If neither antigen, nor antibody is in excess, no precipitate will develop on either plate. The electrophoreses were run for 30 minutes at 10 V per cm, whereafter the gels were washed, dried, and stained as described earlier (10).

Immunofluorescence studies. For these studies it is important to get rid of platelets. Buffy coat leucocytes were isolated from defibrinated blood from normal adults by the method of *Bøyum* (1964) and washed in PBS containing 5 per cent HSA (Cutter, USA). For studies with HSA/anti-HSA complexes, PBS alone was used. Serum was incubated with antigen or particles in the several different combinations and concentrations given in Tables 1 to 7, at 35° C for 10 minutes. The washed cells were added to these mixtures and incubated for 15 minutes at 35° C. After two sets of washing the cells were smeared on glass slides and fixed in 96 per cent ice-cold ethanol for 5 minutes. Direct immunofluorescence was used to study the occurrence of St-Ag, IgG, IgM, IgA, C3 and fibrinogen in the cytoplasm of the cells, using the corresponding FITC-conjugated antisera. Smears were incubated with conjugates for 30 minutes at room temperature in moist chambers, washed two times in PBS for 10 minutes, and mounted in glycerol/PBS solution, 1:2. Fluorescence microscopy was done by immediate reading in a Leitz Orthoplan fluorescence epi-illumination microscope equipped with interference- and glass-filters, as described earlier (30).

Combined NBT and fluorescence studies. 0.2 ml normal serum was incubated with 20 μl St-Ag at 35° C for 10 minutes. Washed blood cells from 0.3 ml heparinized normal blood were added and the mixture incubated with NBT solution as outlined above. After washing in PBS-albumin the cells were fixed, smeared, and incubated with conjugates as described above. The cells were observed by both fluorescence microscopy, and by light microscopy using 500 × magnification.

Statistical calculations. Wilcoxon's test for pair differences (6). Level of significance: 5 per cent.

RESULTS

NBT studies. A. Effect of St-Ag in presence and absence of serum factors. Fig. 1 shows that St-Ag induces a dose-dependent increase

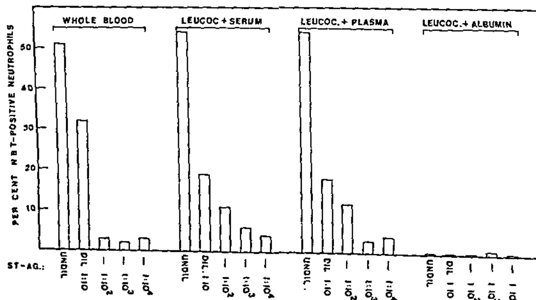


Fig. 1. Effect upon NBT-reduction of St-Ag in the presence and absence of serum factors. One experiment, using a normal serum.

10*

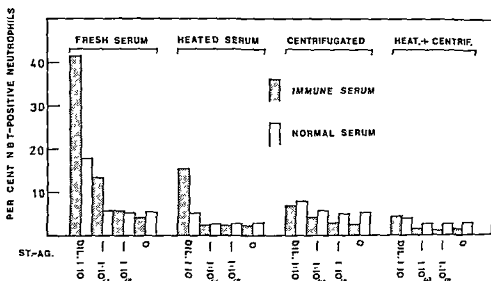


Fig. 2. Effect upon NBT-reduction of St-Ag in combination with fresh and heat-inactivated (56° C/30 min), normal and immune serum, and effect of removal of large-size complexes by high-speed (48,200 × g/30 min) centrifugation. Mean values of 5 experiments, using immune sera (dark columns) and 5 experiments, using normal sera (open columns).

in NBT-reducing neutrophils in the presence of fresh normal serum or plasma, while it fails to do so in the presence of albumin only. Similar results were obtained with immune serum.

B. Effect of St-Ag in combination with fresh and inactivated, normal and immune serum, and effect of removal of large-size complexes. 1) St-Ag (dil. 1:10) caused a higher number of NBT-reducing cells in the presence of immune serum than in the presence of normal serum, as described earlier (15). 2) The response of the neutrophils to this concentration of St-Ag was significantly reduced by a) prior heat-inactivation of serum, and b) centrifugation of the St-Ag/serum mixture, both with immune and with normal serum. 3) The combination of inactivation and centrifugation significantly reduced the effect of immune serum compared to inactivation alone, but not compared to centrifugation alone. Using normal serum this combination did not reduce the effect compared to centrifugation or inactivation alone. 4) In spite of inactivation and centrifugation, the highest concentration of St-Ag still induced significantly more NBT reducing cells than unstimulated controls when immune serum was employed (Fig. 2).

C. Effect of absorption of antibodies to St-Ag. Five experiments, 2 with normal and 3 with immune serum, were done, all showing a consistent pattern; Fig. 3 shows an example, employing a serum with 5 precipitins against St-Ag. 1) Each time St-Ag reacts with fresh serum and is centrifugated, the subsequent addition of more St-Ag to the supernatant becomes less effective in inducing NBT-reduction. 2) As more and more St-Ag is added, centrifugation becomes less and less effective in removing material causing reduction, particularly using inactivated serum. 3) Although fractions I and II from inactivated serum are less effective than the same fractions from fresh serum, then fraction IV and also IVs from inactivated serum are at least as effective as corresponding fractions from fresh serum in causing NBT reduction. After each centrifugation a visible, though diminishing, precipitate was noted and all precipitates were capable of inducing NBT-reduction in washed leucocytes (data not shown).

Immunofluorescence studies. Several ex-

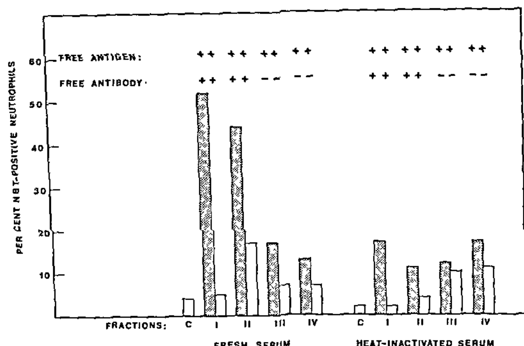


Fig. 3. Effect upon NBT-reduction of absorption of antibodies to St-Ag. Dark columns: after incubation of serum with St-Ag. Open columns: corresponding 48,200 × *g* supernatants. C: unstimulated control values For details, see text. One experiment using immune serum.

TABLE 2. *Specificity of FITC-St-Ab*

Reaction system		Reaction with leucocytes	FITC-conjugate	Intra-cytoplasmic fluorescence (– to + + +)
Source of antibody	Source of antigen			
0.2 ml anti-IgG*	5 µl normal serum	35° C/10 min	St-Ab	–
0.2 ml anti-IgM	100 µl „	„	„	–
0 2 ml anti-IgA	40 µl „	„	„	–
0.2 ml anti-C3	100 µl „	„	„	–
0.2 ml anti-fibrinogen	20 µl „	„	„	–
0.2 ml normal serum	2 µl St-Ag	„	„	+ + +
0 2 ml „	2 µl *E. coli*-Ag	„	„	–
0.2 ml „	2 µl *S. pyogenes*-Ag	„	„	–

* Human IgG, IgM, IgA, C3, and fibrinogen were precipitated with corresponding antisera at point of equivalence, as calculated by affinity-data provided by the manufacturer of the specific antisera (Dakopatts).

periments were done to study the specificity of the FITC-labelled St-Ab. Insolubilized St-Ag stained brightly with FITC-St-Ab, but prior blocking with unconjugated St-Ab markedly reduced this staining. Absorption of FITC-St-Ab with insolubilized St-Ag markedly reduced the ability to stain phagocytized live *Ps. aeruginosa*. The results of further control studies confirming the specificity of FITC-St-Ab are given in Tables 1 and 2.

When immune serum was incubated with St-Ag, and the mixture exposed to neutro-

TABLE 3. *Participation of Immunoglobulin and C3 in Immune Complex Ingestion by Neutrophil Exposed to St-Ag and Normal or Immune Serum*

Reaction system		Reaction with leucocytes	FITC-conjugate	Intracytoplasmic fluorescence (– to + + +)
Source of antibody	*Source of antigen*			
1.0 ml immune serum	10 μl St-Ag	35° C/10 min	St-Ab	+ + +
"	"	"	anti-IgG	+ + +
"	"	"	anti-IgM	+ + +
"	"	"	anti-IgA	+ + +
"	"	"	anti-C3	+ + +
1.0 ml normal serum	"	"	St-Ab	+ + +
"	"	"	anti-IgG	+ +
"	"	"	anti-IgM	+ +
"	"	"	anti-IgA	+
"	"	"	anti-C3	+ +

phils at 35° C, intracytoplasmic localization of St-Ag as well as IgG, IgM, IgA, and C3 could be demonstrated (Table 3), indicating immune complex ingestion. The fluorescence was coarsely granular, suggestive of phagosomes (Fig. 4). Big inclusions often stained "ring-formed", suggesting close association with the phagosome membrane. When normal serum was used, the same phenomena *were observed (Table 3), but the brightness* of fluorescence, the size and number of granules, as well as the number of positive cells was reduced. The latter difference could be visualized semiquantitatively by reducing the amount of added St-Ag (Table 4).

That the observed staining was not caused by simple adherence to the cells, was ruled out in the experiment shown in Table 5, since the granular intracytoplasmic staining was not seen when the experiment was run at 4° C.

In the experiments shown in Table 6, we

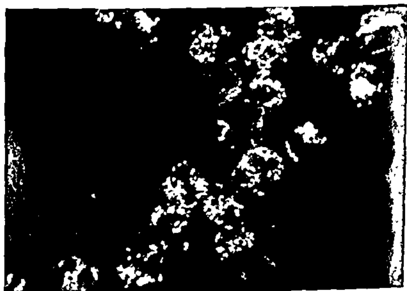


Fig. 4. Immunofluorescence microscopy of neutrophils, incubated with St-Ag and immune serum Stained with rabbit-anti-IgA. 500 ×.

TABLE 4. *Effect of Dilution of St-Ag upon Ingestion of Immunoglobulin by Neutrophils in Normal and Immune Serum*

Reaction system			FITC-conjugate	Intracytoplasmic fluorescence	
Source of antibody	Source of antigen	Reaction with leucocytes		Strength (– to + + +)	Per cent positive neutrophils
0.3 ml immune serum	30 μl St-Ag	35° C/10 min	anti-IgG	+ + +	100
"	"	"	anti-IgM	+ + +	100
"	30 μl St-Ag dil. 1:100	"	anti-IgG	+ + +	100
"	"	"	anti-IgM	+ +	100
"	30 μl St-Ag dil. 1:10,000	"	anti-IgG	+ + +	> 90
"	"	"	anti-IgM	+	> 90
0.3 ml normal serum	30 μl St-Ag	"	anti-IgG	+ + +	> 95
"	"	"	anti-IgM	+ +	> 75
"	30 μl St-Ag dil 1:100	"	anti-IgG	+	< 25
"	"	"	anti-IgM	+ +	< 25
"	30 μl St-Ag dil. 1:10,000	"	anti-IgG	+	< 5
"	"	"	anti-IgM	+	< 5

TABLE 5. *Effect of Temperature upon Ingestion of Immunoglobulin and C3 by Neutrophils Exposed to St-Ag and Serum*

Reaction system			FITC-conjugate	Intra-cytoplasmic fluorescence (– to + + +)	Comments
Source of antibody	Source of antigen	Reaction with leucocytes			
1.0 ml normal serum	10 μl St-Ag	35° C/10 min	anti-IgG	+ + +	Coarse, granular—clearly intracellular
"	"	"	anti-IgM	+ + +	"
"	"	"	anti-IgA	+ +	"
"	"	"	anti-C3	+ + +	"
"	"	4° C/10 min	anti-IgG	–	A weak diffuse staining over entire cell surface
"	"	"	anti-IgM	–	"
"	"	"	anti-IgA	–	"
"	"	"	anti-C3	–	"

studied whether the factors reducing the effect of St-Ag in inducing NBT-reduction, i.e. inactivation of serum or removal of large complexes, would also alter the pattern of fluorescence. Both procedures reduced intracytoplasmic staining of IgG, IgM, and C3. Centrifugation led to much smaller and fewer granules with weaker fluorescence, while inactivation led to fewer granules of sizes comparable to those seen if fresh serum was used, but now devoid of staining with anti-C3.

Table 6 also shows that HSA/anti-HSA complexes, precipitated at point of equivalence, led to the same coarsely granular staining as that observed if St-Ag in combination with fresh serum were used. Complexes, formed at 8 times antigen-excess, were

TABLE 7. *Ingestion of Immunoglobulin and C3 by Neutrophils Exposed to Various other Microbial Antigens and Serum*

Reaction system				
Source of antibody	Source of antigen	Reaction with leucocytes	FITC-conjugate	Intracytoplasmic fluorescence (– to + + +)
0.5 ml normal serum	5 µl *N. meningitidis*-Ag	35° C/10 min	anti-IgG	+ + +
"	"	"	anti-IgM	+ +
"	"	"	anti-IgA	+ +
"	"	"	anti-C3	+ +
"	5 µl *S. pyogenes*-Ag	"	anti-IgG	+ +
"	"	"	anti-IgM	+
"	"	"	anti-IgA	+
"	"	"	anti-C3	+ +
"	5 µl *Staph. aureus*-Ag	"	anti-IgG	+ + +
"	"	"	anti-IgM	+ +
"	"	"	anti-IgA	+ +
"	"	"	anti-C3	+ + +
"	5 µl *D. pneumoniae*-Ag	"	anti-IgG	+ + +
"	"	"	anti-IgM	+ +
"	"	"	anti-IgA	+ +
"	"	"	anti-C3	+ + +

Finally, it was studied whether St-Ag was unique in bringing about ingestion of immunoglobulin and C3. Table 7 shows that 4 similar antigen-preparations, from different species of bacteria, all caused the characteristic intracytoplasmic localization of immunoglobulin and C3. All were also potent stimulators of NBT-reduction (*Koch, C.*: unpublished observation). The possibility that the sonicated extracts might contain enzymes, capable of reducing NBT subsequent to ingestion, was ruled out if neutrophils from 3 patients with chronic granulomatous disease, grossly deficient in ability to reduce NBT were used (2). No farmazan crystals could be detected in such cells, even in the presence of high doses of St-Ag, or other extracts (*Koch, C.*: unpublished observation).

DISCUSSION

The NBT-reduction induced by endotoxin is dose-dependent, is diminished in heat-inactivated serum and by prior absorption of specific antibodies, and is markedly reduced in the absence of serum (18). Stimulation by high doses of endotoxin, in the absence of serum, may be due to direct stimulation of oxydative metabolism (8), since the cell membrane appears to be permeable to NBT (9).

The present experiments show that, when a highly complex microbial extract is used as stimulant, the response of the cells is subject to the same kinetics (Figs. 1 to 3), but we failed to demonstrate "serum-independent" stimulation (Fig. 1). Both with endotoxin and complex microbial antigens, NBT-reduction thus seems to depend upon specific antigen-antibody interaction, prior to reaction with the cells, and this may very likely be true for other microbial products (Table 7 and ref. 14, 17).

Heparin enhances NBT-reduction (4, 18, 25) and it has been suggested that endotoxin causes "serum-independent" stimulation of ingestion—and subsequent intracellular reduction—of NBT complexed to heparin and/or fibrinogen (25). Neither the studies by *Nydegger et al.* (1973 a) on endotoxin, nor the present study of *Ps. aeruginosa*-antigens, support that this should be the more important mechanism. The concentration of residual heparin in the cellular pellet used for mixing

with medium or serum in the experiments shown in Figs. 1 to 3, was unaltered, and we failed to detect fibrinogen in the cells by fluorescence studies after incubation of heparinized whole blood with St-Ag in the presence of NBT.

When St-Ag reacts with immune serum, a number of different antigens can bind to specific antibodies (10, 11, 12), giving rise to multiple points of equivalence and immune complexes of varying size. In the experiments shown in Fig. 3, two things occur: during centrifugation precipitating complexes are removed and, through fractions I to IV, specific antibody is gradually removed. Complete removal of antibody was not achieved since a visible precipitate (containing St-Ag in counterimmunoelectrophoresis) was formed at each addition of St-Ag, but it appears that the complexes become smaller and smaller as the antigen-excess increases, since centrifugation becomes less and less effective in removing complexes causing NBT-reduction.

Although the quantitative distribution between precipitating and soluble complexes in the St-Ag/serum mixture is unknown, it appears likely, from the experiments shown in Figs. 2 and 3, that precipitating complexes *are more potent inducers of NBT-reduction* than soluble complexes. Support for this stems from recent observations, showing that BSA/anti-BSA complexes were potent stimulators of NBT-reduction (*19*). The kinetics of stimulation were similar to the present system—precipitating complexes were more effective than soluble ones, and heat-inactivation or interference with complement-fixation of the complexes reduced the effect. Furthermore removal of the Fc-portion of the antibody, which would prevent attachment of the complexes to the cells, abolished the effect (19).

None of these studies answer the question whether NBT-reduction in these situations depends upon ingestion of complexes. In our studies, the application of direct immunofluorescence technique demonstrates that antigen, as well as immunoglobulin and C3, was in fact ingested when the cells were exposed to the St-Ag/serum mixture under conditions leading to increased NBT-reduction (Table 3). Moreover, formazan crystals were only found in cells showing such ingestion (Fig. 5). The finding that IgG, as well as IgM and IgA, was ingested, would indicate ingestion either of different complexes involving each of these Ig-classes or of mixed complexes involving St-Ag molecules with *many antigenic determinants, binding* specific antibodies belonging to separate Ig-classes. It should, however, also be considered whether mixed complexes could be formed by the interaction of specific antigen/antibody complexes with "naturally" occurring antiglobulins or immunoconglutinins, so commonly found in the sera of infected patients (3, 16).

It is interesting that normal serum, as well as immune serum, was capable of forming large, easily ingested, complexes with St-Ag, since the crossed immunoelectrophoresis technique failed to detect precipitating antibodies against St-Ag in normal serum. Using more sensitive methods, however, it has later been shown (13) that more than 50 per cent of normal sera, as well as commercial gammaglobulin, contain low-titred precipitins against St-Ag. *These precipitins are, however,* cross-reactive and not specific to *Ps. aeruginosa* (12, 13), in contrast to the precipitins demonstrated in the patients in these studies (*Høiby, N.: unpublished observations*). This suggests a very important role of these low-titred, "naturally" occurring, cross-reactive antibodies—namely the ability to form large, possibly mixed, complexes *with microbial* antigens, promoting stimulation, followed by rapid clearing by phagocytic cells of toxic material or material otherwise damaging to the host. Moreover, as mentioned *above*, "naturally" occurring antiglobulins and/or immunoconglutinins may play a critical physiological role in promoting the formation of such large complexes. This might *diminish* the risk that bacterial infections should give rise to circulating soluble complexes, less easily cleared from the circulation, which would be highly important in view of *the*

possible damaging effect of such complexes (22).

These studies do not explain the increased spontaneous NBT-reduction displayed by a high number of circulating neutrophils from patients with bacterial infections (17, 20) since we have so far been unable to detect intracytoplasmic immunoglobulin in neutrophils from such patients. The phenomenon of immune complex ingestion, demonstrated *in vitro* in these studies, however, may occur *in vivo* during bacterial infection if cells, having ingested such complexes, are cleared from the circulation too rapidly to be detected in an appreciable number. Other mechanisms, currently under investigation, may therefore also be taken into account for the *in vivo* activation of neutrophils during bacterial infections.

We express our gratitude to Professor *Viggo Faber* and Dr. *Klaus Jensen*, for helpful advice and criticism. Mrs. *Annie Bethien*, and Mrs *Ulla Høiby* are thanked for skilful technical assistance.

This study was aided by grants from *The Michaelsen Foundation, The Thorvald Madsen Legat, The National Danish Association against Rheumatic Diseases, The National Danish Association against Cystic Fibrosis*, and *The Danish Medical Research Council*.

REFERENCES

1. *Avrameas, S. & Ternynck, T.*: The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents Immunochemistry 6: 53–66, 1969.
2. *Baehner, R. L. & Nathan, D. G.*: Quantitative nitroblue tetrazolium test in chronic granulomatous disease. New Engl. J Med 278: 971–976, 1968.
3. *Bartfeld, H*: Distribution of rheumatoid factor activity in nonrheumatoid states. Ann. New York Acad. Sci. *168*: 30–38, 1969.
4. *Björkstén, B*: The influence of technical factors on the NBT test. Scand. J. Haemat. *12*: 46–50, 1974
5. *Bøyum, A.*: Separation of white blood cells. Nature (Lond) *204*: 793–794, 1964.
6. *Diem, K* (Edit.): Documenta Geigy Scientific Tables. J. R. Geigy S. A., Basel 1962 p. 128
7. *Duquesnoy, R J*: Rapid quantitation of the immunoprecipitin reaction in agarose gel by electroimmunodiffusion with paper discs. J. Immunol. Meth. *3*: 221–226, 1973.
8. *Graham, R. C., Karnowsky, M. J., Shafer, A. W., Glass, E. A. & Karnowsky, M. L.*: Metabolic and morphological observations on the effect of surface-active agents on leucocytes. J. Cell Biol. *32*: 629–647, 1967.
9. *Humbert, J. R., Gross, G. P., Vatter, A. E. & Hathaway, W. E.*: Nitroblue tetrazolium reduction by neutrophils: biochemical and ultrastructural effects of methylene blue. J. Lab. Clin. Med. 82: 20–30, 1973.
10. *Høiby, N. & Axelsen, N. H.*: Identification and quantitation of precipitins against *Pseudomonas aeruginosa* in patients with cystic fibrosis by means of crossed immunoelectrophoresis with intermediate gel. Acta path. microbiol. scand. Sect. B, *81*: 298–308, 1973.
11. *Høiby, N.*: *Pseudomonas aeruginosa* infection in cystic fibrosis. Relationship between mucoid strains of *Pseudomonas aeruginosa* and the humoral immune response. Acta path. microbiol. scand. Sect. B, *82*: 551–558, 1974.
12. *Høiby, N.*: Cross-reactions between *Pseudomonas aeruginosa* and thirty-six other bacterial species. Scand. J. Immunol. Suppl. 2, 1975. In press.
13. *Høiby, N.*: Normally occurring precipitating antibodies against *Pseudomonas aeruginosa*. Prevalence, specificities and titres. Scand. J. Immunol. Suppl. 2, 1975. In press.
14. *Koch, C.*: Studies on the nitroblue-tetrazolium staining induced in human neutrophils by bacterial products. Acta path. microbiol. scand. Sect. B, *81*: 266–268, 1973.
15. *Koch, C. & Høiby, N.*: Nitroblue-tetrazolium staining of human neutrophil granulocytes. Effects of stimulation with *Pseudomonas* antigens in the presence or absence of human Pseudomonas precipitins. Acta path. microbiol. scand. Sect. B, *81*: 787–794, 1973.
16. *Lachmann, P. J.*: Conglutinin and immunoconglutinins. Advan Immunol. *6*: 479–527, 1967.
17. *Matula, G. & Paterson, P. Y.*: Spontaneous in vitro reduction of nitroblue tetrazolium by neutrophils of adult patients with bacterial infection. New Engl. J. Med. *285*: 311–317, 1971.
18. *Nydegger, U. E., Miescher, A., Anner, R. M., Creighton, D. W., Lambert, P. H. & Miescher, P. A.*: Serum and cellular factor involvement in nitroblue tetrazolium (NBT) reduction by human neutrophils. Klin. Wschr. *51*: 377–382, 1973 a.
19. *Nydegger, U. E., Anner, R. M., Gerebtzoff, A., Lambert, P. H. & Miescher, P. A.*: Polymorphonuclear leucocyte stimulation by immune complexes. Assessment by nitroblue te-

trazolium reduction. Eur. J. Immunol. *3.* 465–470, 1973 b.

20. *Park, B. H., Fikrig, S. M. & Smithwick, E. M.:* Infection and nitroblue-tetrazolium reduction by neutrophils. Lancet *II:* 532–534, 1968.
21. *Park, B. H. & Good, R. A.:* NBT test stimulated. Lancet *II:* 616, 1970.
22. *Peters, D. K. & Lachmann, P. J.:* Immunity deficiency in pathogenesis of glomerulonephritis. Lancet *I:* 58–60, 1974.
23. *Rhodes, J. M.:* In vitro studies on the fate of antigen. IV The fate of soluble and precipitated antigen antibody complexes in the presence of guinea pig exudate cells, correlated with their skin reactivity. Int. Arch. Allergy Appl. Immunol. *25:* 225–241, 1964.
24. *Segal, A. W., Trustey, S. F. & Levi, A. J.:* Re-evaluation of nitroblue-tetrazolium test. Lancet *II:* 879–883, 1973.
25. *Segal, A. W. & Levi, A. J.:* The mechanism of entry of dye into neutrophils in the nitroblue tetrazolium (NBT) test. Clin. Sci. Mol. Med. *45:* 817–826, 1973.
26. *Sewell, M. M. H.:* A semi-quantitative technique using the LKB immunodiffusion apparatus. Science Tools *14:* 11–12, 1967.
27. *Steigbigel, R. T., Johnson, P. K. & Remington J. S.:* The nitroblue tetrazolium reduction test versus conventional hematology in the diagnosis of bacterial infection. New Engl. J. Med. *290:* 235–238, 1974.
28. *The, T. H. & Feltkamp, T. E. W.:* Conjugation of fluorescein isothiocyanate to antibodies I. Experiments on the conditions of conjugation. Immunol. *18:* 865–873, 1970.
29. *Wiik, A. & Munthe, E.:* Restrictions among heavy and light chain determinants of granulocyte-specific antinuclear factors. Immunol. *23:* 53–60, 1972.
30. *Wiik, A., Jensen, E. & Friis, J.:* Granulocyte-specific antinuclear factors in synovial fluids and sera from patients with rheumatoid arthritis. Ann. Rheum. Dis. *33.* 515–522, 1974.

Acta path. microbiol. scand. Sect. C, *83:* 157–164, 1975

MIGRATION INHIBITORY FACTOR (MIF) AND THE HUMAN MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

S. Stenbjerg and K. Mygind

Blood Bank and Tissue Typing Laboratory, University Hospital, Aarhus, Denmark

Stenbjerg, S. & Mygind, K. Migration inhibitory factor (MIF) and the human major histocompatibility complex (MHC). Acta path. microbiol. scand. Sect. C, *83:* 157–164, 1975.

MIF assay was performed on cell free supernatants from mixed leucocyte cultures (MLC) between 1) HL-A identical siblings, 2) HL-A haploidentical related persons, and 3) HL-A unidentical, unrelated persons. It was found that 1) MLCs between HL-A identical siblings produced no MIF, 2) HL-A haploidentical related combinations released 1/3–1/4 the amount of MIF as compared to HL-A unidentical, unrelated combinations. It was concluded that the release of MIF in MLC is governed by the MHC chromosomal region and that estimation of MIF quantities produced in MLC may be of value in histocompatibility testing. Mitomycin C treated MLCs released MIF indicating that MIF production in the MLC is independent of DNA synthesis.

Key words: Migration inhibitory factor; histocompatibility.

S. Stenbjerg, Blood Bank and Tissue Typing Laboratory, University Hospital, Aarhus, Denmark.

Received 26.x.74 Accepted 8.xi.74

When confronted with the appropriate antigen, sensitized lymphocytes are known to liberate several substances, lymphokines, among which lymphocytotoxic factor (11), mitogen factor (9), and *m*igration *i*nhibitory *f*actor (MIF) (7, 19) have been most extensively investigated. Non-sensitized lymphocytes stimulated by phytohaemagglutinin and concanavalin A release factors with similar characteristics (5, 10, 13). Substances with the same activities have been demonstrated in the supernatants from mixed leucocyte cultures (MLC) (2, 6, 12, 15, 18), but none of these reports have dealt with the genetics of the MIF production.

The extent of compatibility in regard to the *m*ajor *h*istocompatibility *c*omplex (MHC), as defined by LD determinants (*l*ymphocyte *d*efined) and SD determinants (*s*erologically *d*efined), seems to govern the MLC activation (1). The production of MIF in the MLC might be dependent on the same chromosomal segment.

To investigate this we have compared the amount of MIF demonstrable in supernatants from MLCs between 1) HL-A identical siblings, 2) persons being HL-A haploidentical by descent, and 3) unrelated HL-A unidentical persons.

MATERIALS AND METHODS

HL-A typing. HL-A typing was performed using the lymphocytotoxic micromethod described by *Kissmeyer-Nielsen & Kjerbye* (16) and the platelet complement fixation test (*Colombani et al.*

...

(8)). HL-A typing of the related persons included family studies allowing hapotype identification.

MLC-technique (modified after *Jørgensen et al.* (14)). Defibrinated blood was diluted with equal amounts of bicarbonate and Hepes buffered medium TC 199 (pH 7.2–7.4) (Flow Lab., Scotland). The lymphocytes were harvested in TC 199 after centrifugation on a Ficoll-Isopaque gradient (Lymphoprep, Nyegaard, Oslo) at 1000 × *g* for 20 minutes at room temperature. The cells were washed once at 500 × *g* for 10 minutes and resuspended in bicarbonate buffered medium RPMI 1640 (Flow Lab., Scotland) with 25 per cent human serum from a pool of approximately 25 male blood donors. Cell concentration was adjusted to 10^6 per ml.

Half of the cells was resuspended in TC 199 containing mitomycin C (Sigma Chem. Comp., St. Louis, USA) 50 µg per ml at a cell concentration of approximately 2 × 10^6 per ml. After incubation for 30 minutes in a 37° C waterbath the cells were washed twice in TC 199 and resuspended in serum-RPMI at 10^6 cells per ml. Mitomycin-treated cells are indicated with the suffix "m".

A given MLC experiment between cells from persons A and B included: two-way cultures (A + B), single cell control cultures (A and B), cultures where both partners were mitomycin-treated (A_m + B_m), corresponding controls (A_m and B_m), and one-way cultures (A + B_m and B + A_m) with autologous controls (A + A_m and B + B_m).

The cultures were incubated in sterile 15 ml round-bottomed glass tubes with loose covers, culture volume (1 ml) was composed of 0.5 ml of each cell suspension or 1 ml single cell suspension (macrocultures). Incubation took place for 54 hours at 37° C in a humid atmosphere of 95 per cent air and 5 per cent CO_2.

The MLC activity in the macrocultures was measured (after 54 hours incubation), i.e.: duplicates of 100 µl resuspended culture with addition of 0.04 µC thymidine-2-^{14}C (Amersham) (in 20 µl normal saline) were incubated for 24 hours at 37° C in round-bottomed Linbro plates (IS-MRC-96-TC). The reaction was stopped by transferring the plates to 4° C. The cultures were harvested using a suction filter device (14) and scintillation counting was performed in a Packard 2450 spectrometer using 1 ml of Instagel® (Packard) as scintillator.

The MLC results were recorded as counts per minute (cpm) (corrected for background) per 0.1 × 10^6 cells and expressed as stimulation ratio (SR): the ratio between cpm of a given two-way culture (A + B) and the mean cpm of the corresponding single cell control cultures (A and B).

After removal of cell suspension for measuring the MLC activity (as described above), the macrocultures were centrifuged at 2000 × *g* for 20 minutes and the cell free supernatants were stored at –20° C until assayed for MIF activity.

Each MLC experiment included a minimum of one related combination (cells from two persons sharing one or two MHC haplotypes: parent/child or sibling/sibling) and two unrelated combinations (cells from each of the related persons cultured with cells from a person being unrelated and sharing no HL-A determinants with the related persons).

Exceptions to this was MLC No. 4 (Table 2) in which the unrelated persons shared two HL-A determinants with one of the related persons, and MLC experiments No. 6 and 8 (Table 2) in which a two haplotype unidentical sibling served as control.

MIF assay. The MIF assay was performed in agarose medium as described by *Clausen* (6).

Isolation of migratory cells, incubation and calculation were performed as described by *Mygind* & *Stenbjerg* (19) with the following modifications: cells were resuspended in serum-RPMI in stead of TC 199 using double cell concentration (4 × 10^6 per ml). 10 µl cell suspension was mixed with 10 µl MLC-supernatant or supernatant diluted in serum-RPMI (final dilutions 1:2 to 1:64) and incubated in Linbro plates for 30 minutes followed by resuspension after 15 minutes. Finally, duplicate cultures of 7 µl were made from each incubation mixture in agarose medium.

Supernatants from the two-way MLCs and from single cell control cultures were assayed. In some cases, also cultures in which both cell compartments had been treated with mitomycin C, and

TABLE 1 *HL-A Genotypes and ABO Phenotypes of 10 Pairs of HL-A Identical Siblings. The Stimulation Ratio of Two-way MLCs Are Given*

MLC No.	MLC reactants: HL-A type	MLC reactants: ABO type	Stimulation ratio
1	1, 8/1, 8	O/O	1.0
2	W19, W5/11, W5	O/O	1.5
3	1, 8/2, W15	O/A	1.2
4	1, 8/2, W15	A/O	1.1
5	1, 8/2, W15	AB/O	0.9
6	1, 8/2, W15	A/AB	1.0
7	W19/2, W15	A/B	1.0
8	1, 8/2, W15	O/AB	1.5
9	1, 8/2, W15	O/O	1.4
10*	W28, W10/2, W17	O/O	/

* MLC No. 10 was performed simultaneously with MLC No. 8 in Table 2 and unfortunately cpm were lost. However, they showed non-stimulation

mitomycin C treated single cell control cultures were tested for MIF activity.

Each supernatant was tested on cells from 2–4 persons, usually male blood donors, and the mean of the MIs recorded for these cells is presented as the final MI.

In a few cases, cells from the MLC reactants were used as migratory cells in the MIF assay, resulting in MIs not differing from those obtained if randomly selected cells were used.

Calculation of Migration Index (MI)

Example 1:

MIF assay of the supernatant from a two-way MLC culture (A + B) using cells from person A and person B.

The migration area of cells incubated in (A + B) supernatants divided by the mean of the migration areas of cells incubated in the supernatants from single cell control cultures (A and B). This ratio was multiplied by 100 to obtain the MI.

$$MI = \frac{\text{Migration area } (A + B)}{\frac{1}{2}\,(\text{Migration area } (A) + \text{Migration area } (B))} \times 100$$

Example 2:

MIF assay of the supernatant from a MLC culture in which both cell compartments have been treated with mitomycin C, ($A_m + B_m$).

The migration area of cells incubated in ($A_m + B_m$) supernatant divided by the mean of the migration areas of cells incubated in the supernatants from single cell control culture (A_m) and (B_m).

$$MI = \frac{\text{Migration area } (A_m + B_m)}{\frac{1}{2}\,(\text{Migration area } (A_m) + \text{Migration area } (B_m))} \times 100$$

Example 3:

MIF assay of the supernatants from single cell control cultures (A) or (B).

The migration area of cells incubated in the supernatant from single cell control culture (A) divided by the migration area of cells incubated in serum-RPMI.

$$MI = \frac{\text{Migration area } (A)}{\text{Migration area (serum-RPMI)}} \times 100$$

RESULTS

Preliminary studies of the time-related MIF kinetics revealed that supernatants from 24h MLC cultures did not inhibit cell migration neither in the related nor the unrelated combinations. At 48 hours there was always some MIF activity, but the activity disappeared after 2–4 fold dilution. The shortest incubation time allowing substantial dilution of the supernatants was 54 hours in the related as well as the unrelated combinations. The investigations also included 72h cultures which produced MIF quantities exceeding those of 54h cultures. For practical reasons, all macro MLCs with cells from HL-A haploidentical persons and control cultures were run for 54 hours. However, all MLC experiments with cells from HL-A identical siblings were incubated for 72 hours.

In a few cases, the supernatants were tested for nonspecific cytotoxicity (12) against a panel of cells from 11 persons. After one hour of incubation, cell viability was between 98 and 100 per cent and, after 24 hours of incubation at 37° C, cell viability was 95 per cent or better and not differing from the values obtained after incubation with serum-RPMI.

The serum from the three mothers used in MLCs No. 1, 3, and 9 (Table 2) did not contain cytotoxic HL-A antibodies and from this it was assumed that the mothers were not immunized within the sensitivity of the method used (16).

1. MIF Assay of MLC Supernatants from a Series of Experiments with Cells from HL-A Identical Siblings

Fig. 1 demonstrates the result of MIF assay of supernatants from MLCs between HL-A identical siblings. Each experiment included an HL-A unidentical third party control.

MLCs between cells from HL-A identical siblings including ABO compatible as well as incompatible combinations (Table 1) did not result in MIF production, while the control MLCs with cells from third party produced considerable amounts of MIF (Fig. 1).

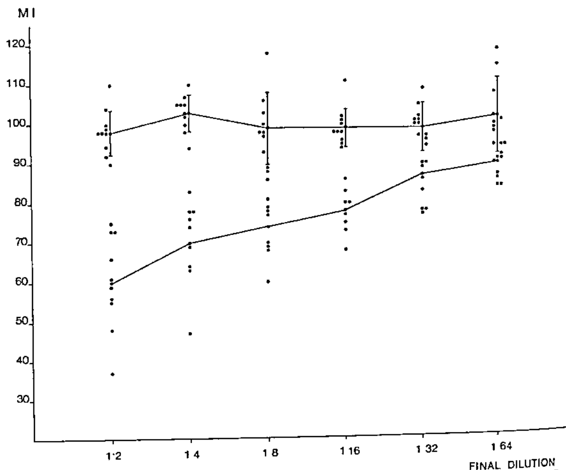


Fig. 1. The MIs obtained from 10 supernatants from MLCs between HL-A identical siblings (●). One standard deviation is indicated. The MIs of the control group of unrelated combinations are shown (○). Ordinate: MI. Abscissa: final dilution of supernatants.

The HL-A genotypes of the HL-A identical siblings are presented in Table 1. Four families were included and one of these had several HL-A identical siblings (MLC Nos. 3–9). The stimulation ratio of two-way MLCs between HL-A identical siblings never exceeded 1.5.

2. *MIF Assay of MLC Supernatants from a Series of HL-A Haploidentical Related Combinations and HL-A Unidentical Unrelated Combinations*

The results of MIF assay of serially diluted two-way MLC supernatants are presented in Table 2.

The unrelated MLC combinations produced greater amounts of MIF than the related haploidentical combinations, resulting in lower MIs in the unrelated group (mean MI 53, dilution 1:2) than in the related (mean MI 66, dilution 1:2). The dilution curve (Fig. 2) demonstrates that the unrelated combinations produce MIF quantities surpassing the amount produced by the related combinations by a factor 3–4 as judged from the supernatant dilutions giving the same MI.

The MIs recorded for each group at all dilution steps were normally distributed (Kolmogorov-Smirnov test) and the results applying to the related and unrelated combinations differed significantly (student t-test) except for 1:64 (p-values are given in Fig. 2).

TABLE 2. *HL-A Type of MLC Reactants (Left Column). The MIs Obtained by the Cell Free MLC Supernatants (Final Dilution 1:2–1:64) Are Presented in the Right Column*

C	HL-A types of MLC reactants		MLC combi-nations	MI of supernatant dilution 1:2–1:64						MLC stim. ratio
				2	4	8	16	32	64	
	1, 12/3, 7*	W19, 5/1, 12	m-c§	67	74	81	94	–	96	18.3
	1, 12/3, 7	2, W28, 8, W10	m-u	70	66	78	83	87	97	21.2
	W19, 5/1, 12	2, W28, 8, W10	c-u	66	85	86	82	95	108	23.0
	2, W10/W19, 27	2, W10/W19, 7	f-c	61	80	85	88	93	100	12.9
	2, W10/W19, 27	1, 8/1, 5	f-u	45	63	67	81	105	103	16.6
	2, W10/W19, 7	1, 8/1, 5	c-u	41	59	79	74	93	97	11.9
	2, 27/11, W5	3, 7/11, W5	m-c	44	61	74	84	91	98	7.3
	2, 27/11, W5	1, 2, 8, 13	m-u	35	44	55	67	80	98	10.7
	3, 7/11, W5	1, 2, 8, 13	c-u	40	37	62	72	85	104	8.0
	3, W15/1, 8	3, W15/W28, W10	s-s	74	95	89	88	95	102	10.9
	3, W15/1, 8	1, 9, 5, 8	s-u	70	73	92	88	99	108	9.5
	3, W15/W28, W10	1, 9, 5, 8	s-u	53	59	61	79	86	95	16.1
	10, W18/1, 8	10, W18/2, W5	f-c	84	101	95	116	112	97	20.6
	10, W18/1, 8	1, 12/3, 7	f-u	65	71	83	95	100	95	24.6
	10, W18/2, W5	1, 12/3, 7	c-u	48	47	72	88	90	99	44.1
5	1, W17/2, 8	9, 12/2, 8	s-s	98	108	100	99	106	–	7.7
	9, 12/W19, W21	9, 12/2, 8	s-s	69	80	92	97	101	–	20.0
	1, W17/2, 8	9, 12/W19, W21	s-s	75	81	94	96	105	–	18.9
7	9, 12/1, 13	9, 12/2, W10	s-s	85	82	117	100	100	105	11.0
	9, 12/1, 13	3, W19, 5, 8	s-u	58	73	69	88	90	93	35.6
	9, 12/2, W10	3, W19, 5, 8	s-u	62	78	81	81	90	111	40.2
8	W28, W10/3, 7	W28, W10/2, W17	s-s	39	62	69	86	89	98	$
	W28, W10/2, W17	2, 7/2, W17	s-s	40	57	87	92	100	–	
	W28, W10/2, W17	3, 7/11, W5	s-u	37	47	60	68	83	94	
	W28, W10/3, 7	2, 7/2, W17	s-s	37	44	61	74	83	87	
9	1, 8/2, W5	1, 8/1, 8	m-c	49	57	74	87	94	89	6.6
	1, 8/1, 8	2, 3, W14, W15	c-u	35	43	48	70	82	91	18.7
10	1, 8/10, W18	1, 8/1, 8	f-c	82	87	94	104	98	102	12.0
	1, 8/1, 8	2, 11, W5, W10	c-u	65	77	83	83	94	115	14.7
	Mean of related combinations:			66	79	88	95	98	99	12.7
	Mean of unrelated combinations:			53	62	72	81	91	100	20.9

* If family studies were included, the HL-A haplotypes are indicated (–/–).

§ The MLC combinations are coded as follows: mother (m), father (f), child (c), sibling (s), and unrelated person (u)

$ Unfortunately the cpm of MLC experiment No. 8 were not stored. They did not, however, deviate from the pattern recorded from the remaining MLCs.

The MLC activity expressed by SR seemed to some degree correlated to the MIF production in the two groups. SR of the unrelated combinations averaged 20.9, while the SR of the related combinations averaged 12.7 (Table 2).

Unstimulated single cell control cultures did not release MIF during 54 hours (or 72 hours) since the MIs of these supernatants ranged between 90–110 using serum-RPMI incubated in parallel with the cell cultures as reference.

the reduced cell viability (average 45 per cent living cells). Besides, impaired protein synthesis due to the high concentration of mitomycin C may have contributed (3, 17, 20).

From this it can be deduced that MIF production in the MLC is dependent on cellular recognition of a determinant (or determinants) governed by the MHC chromosomal region, but independent of DNA synthesis. However, *it remains to be established which* part of the MHC chromosomal region governs MIF production. If not due to insensitivity of the test system, the result of MLC 6 (Table 2) in which a haploidentical pair of siblings *did not produce MIF in two experiments in* spite of stimulation in MLC, could indicate that determinants other than SDs and LDs are involved.

Studies further elucidating the genetics of *MIF production in MLC are in progress.*

The study was aided by grants from the *Danish Medical Research Council* (No. 512-2811).

REFERENCES

1. *Bach, F. H.:* The major histocompatibility complex in transplantation immunology. Transpl. Proc. *5:* 23–29, 1973.
2. *Bartfeld, H. & Atoynatan, T.:* Activity and properties of macrophage migration inhibitory factor produced by mixed lymphocyte cultures. Nature *230:* 246–247, 1971.
3. *Bloom, B. R. & Bennett, B.:* Migration inhibitory factor associated with delayed-type hypersensitivity. Fed. Proc. *27: 13–15, 1968.*
4. *Bloom, B. R , Gaffney, J. & Jimnez, L.*· Dissociation *of MIF production and cell pro*liferation. J. Immunol. *109.* 1395–1398, 1972.
5. *Christoforetti, R. & Fireman, P.: Studies* of a non-cytotoxic migration inhibitory factor from human leucocytes Nature *241* · 219–221, 1973.
6. *Clausen, J E..* Migration inhibitory effect of cell-free supernatants from mixed human lymphocyte cultures. J. Immunol. *108:* 453–459, 1972.
7. *Clausen, J. E .* Tuberculin-induced migration inhibition of human peripheral leucocytes in agarose medium Acta Allergol. *26:* 56–80, 1971.
8. *Colombani, J , D'Amaro, J , Gabb, B., Smith, G. & Svejgaard, A..* International agreement on a microtechnique of platelet complement fixation (Pl. C Fix). Transpl. Proc. *3:* 121–*126, 1971.*
9. *Dumonde, D. C., Howson, W. T. & Wolstencroft, R. A.:* The role of macrophages and lymphocytes in reactions of delayed hypersensitivity. In: Miescher, P. A. & Grabar, P. (Eds.): Immunopathology. Fifth International symp. *1967.* Schwabe, Basel *1968,* pp. 263–280.
10. *Granger, G. A., Moore, G. E., White, J. G, Matzinger, P., Sundsmo, J. S., Shupe, S, Kolb, W. P., Kramer, J. & Glade, P. R.:* Production of lymphotoxin and migration inhibitory *factor by established human lympho*cytic cell lines. J. Immunol. *104:* 1476–1485, 1970.
11. *Granger, G. A., Schacks, S. J., Williams, T. W. & Kolb, W. P.:* Lymphocyte in vitro cytotoxicity: Specific release of lymphotoxin-like materials from tuberculin-sensitive lymphoid cells. Nature *221:* 1155–1157, 1969.
12. *Granger, G. A. & Williams, T. W.:* Lymphocyte cytotoxicity in vitro: Activation and release of a cytotoxic factor. Nature *218:* 1253–1254, 1968.
13. *Horvat, M., Havemann, K., Sodomann, C. P. & Bürger, S.:* Mitogenic factor and migration inhibitory factor in supernatants of serum-free human lymphocyte cultures stimulated with concanavalin A. Int. Arch. Allergy *43:* 446–456, 1972.
14. *Jørgensen, F., Lamm, L. U. & Kissmeyer-Nielsen, F.:* Mixed lymphocyte cultures with inbred individuals. Tissue Antigens *3:* 323–339, 1973.
15. *Kasakura, S. & Lowenstein, L ·* A factor stimulating DNA synthesis derived *from the me*dium of leucocyte cultures. Nature *208:* 794–*795, 1965.*
16. *Kissmeyer-Nielsen, F. & Kjerbye, K. E.: Lymphocytotoxic microtechnique.* Purification of lymphocytes by flotation. In· Curtoni, E. S, Mattuiz, P. L. & Tosi, R. M. (Eds.): Histocompatibility Testing. Munksgaard, Copenhagen, 1967, pp. 381–383.
17. *Mizoguchi, Y., Yamamoto, S & Morisawa, S:* Studies on the biosynthesis of macrophage migration inhibitory factor in delayed hypersensitivity. J Biochem. *73:* 467–474, 1973
18. *Mookerjee, B. K., Lao, V S. H , Pabst, H. D. & Dossetor, J. B.:* In vitro detection of cellular hypersensitivity to HL-A antigens *in man.* Proceedings of the seventh leucocyte culture conference, 613–636, Academic *Press,* New York, 1973.
19. *Mygind, K. & Stenbjerg, S* Leucocyte migration test in agarose. Acta path. microbiol. scand. Sect. B, *82:* 277–282, 1974.
20. *Shatkin, A. J., Reich, E., Franklin, R. M. & Tatum, E. L.:* Effect of mitomycin C on mammalian cells in culture. Biochem. Biophys. Acta *55:* 277–289, 1962.

Acta path. microbiol. scand. Sect. C, *83:* 165–172, 1975

FURTHER EVIDENCE OF AN EXTENSIVE CROSS-REACTIVITY BETWEEN THE THREE MAIN PARTS OF THE BOVINE ENCEPHALITOGENIC PROTEIN IN THE LYMPHOID CELL TRANSFORMATION TEST AND THE MACROPHAGE MIGRATION INHIBITION TEST

HÅKAN BERGSTRAND and BENGT KÄLLÉN

Research Laboratories of AB Draco, Lund, and Tornblad Institute, University of Lund, Sweden

Bergstrand, H. & Källén, B. Further evidence of an extensive cross-reactivity between the three main parts of the bovine encephalitogenic protein in the lymphoid cell transformation test and the macrophage migration inhibition test. Acta path. microbiol. scand. Sect. C, *83:* 165–172, 1975.

The results presented indicate that the three main peptide parts of bovine encephalitogenic protein (BEP) cross-react when examined by the macrophage migration inhibition test in guinea pigs and the lymph node cell transformation test in rabbits: 1. Guinea pigs were immunized with peptide 43–88 of BEP and the capillary migration of peritoneal cells from these animals was examined in the presence of the intact protein or various peptide fragments derived from the latter. The complete protein and peptides 1–42 and 92–169 were all capable of inhibiting cell migration, but none of these antigens were as efficient as the immunizing agent (peptide 43–88). 2. Lymph node cells from rabbits sensitized with peptide 1–42, 43–88, or 92–169 were examined using the lymphoid cell transformation test. Cells from animals immunized with any one region always showed a substantial degree of transformation also when stimulated by peptides covering the other two regions of the protein, but the immunizing peptide consistently showed a transforming effect better than that of the other two peptides. 3. Control experiments showed that contamination of the immunizing peptide with other parts of the protein could not explain the observed cross-reactivity between the three main regions of BEP.

Key words: Bovine encephalitogenic protein; cross-reactivity.

H. Bergstrand, AB Draco, Fack, S-221 01 Lund 1, Sweden.

Received 25.ix.74 Accepted 11.xi.74

Studies of cell-mediated immunity using chemically well-defined immunogens of low molecular weight such as derivatives of dinitrophenyl-oligo-lysines, have revealed an extremely stringent specificity for this type of immunity which in some instances seems comparable to or even better than that of

humoral antibodies. On the other hand, several investigators employing various *in vivo* or *in vitro* techniques for estimating cell-mediated immunity report findings that indicate the existence of an unexpected degree of cross-reactivity between several antigens, mostly of protein nature. In many instances, this cross-reactivity cannot be simultaneously demonstrated on the level of humoral immunity (for further references, cf. *Rajewsky* & *Mohr* 1974 and *Weinbaum et al.* 1974). The macrophage migration inhibition (MMI) test, in its direct form usually performed with peritoneal cells from sensitized guinea-pigs, and the lymphoid cell transformation (LT) test are considered two of the most reliable *in vitro* correlates of cell-mediated immunity, although neither seems quite specific for this kind of immunity. We have previously reported studies in which these techniques were used and primarily aimed at a localization of antigenic determinants on the basic encephalitogenic protein of central nervous system myelin—the causative agent in experimental allergic encephalomyelitis. During this work, evidence was obtained indicating a substantial degree of "cross-reactivity" between two parts of the protein molecule (*Bergstrand* & *Källén* 1973a). The phenomenon could not unequivocally be considered due to immunological cross-reactivity based on structural similiarities between different antigenic determinants, although the evidence appeared to favour that explanation The possibility that studies of the antigenic specificity in cell-mediated immunity might have some bearing on a) the autoimmune effects exerted by this protein and b) the still highly controversial issue of the specificity and the nature of the recognition units in cell-mediated immunity warranted further experiments with these peptides, aimed at examination of the basis of the "cross-reactivity" phenomenon.

MATERIAL AND METHODS

Migration Inhibition Experiments

Normal outbred guinea-pigs were sensitized with a peptide constituting region 43–88 of the bovine encephalitogenic protein (BEP) (figures indicate amino acid residue number according to the revised sequence given by *Brostoff et al.* 1974). Each animal was injected with 0.05 ml of an emulsion of equal volumes of Freund's complete adjuvant (FCA) and peptide solution into each of three foot pads, the total dose given to each animal being 25 µg of peptide 43–88. The animals were injected intraperitoneally with 20 ml of sterile paraffin oil 7 days after sensitization. The peritoneal cells were harvested 3–10 days later and the migration test was performed as previously described (*Bergstrand* & *Källén* 1972) using disposable micropipettes (25 µl, Drummond Sci. Corp) as capillary tubes. The migration of the cells was recorded by tracing the cell fan on transparent paper after 18–20 hours of incubation and planimetrial measurement of the area.

Lymphocyte Transformation Test

Methodological details are given in earlier papers (cf. *Bergstrand* & *Källén* 1973b). Rabbits were immunized by injection into three foot pads of peptides derived from BEP emulsified in Freund's complete adjuvant. Lymphocytes were obtained from regional lymph nodes and cell cultures were set up with various antigens added and cultured for 3 days. Lymphocyte transformation (LT) was assessed by incorporation of tritiated thymidine and liquid scintillation counting. In order to obtain variates with near-normal distribution, c.p.m. values were transformed to $\log_{10}$ c.p.m. (cf. *Bergstrand* & *Källén* 1973b).

Antigens

The preparation of BEP and the various peptides from the latter as well as the methods used for control of their purity have been described previously (*Bergstrand* 1971, 1973) The following peptides were used in the present study: 1–42, 43–88, 43–115, tyr, 92–169, HNB-92–169 (i.e. peptide 92–169 with the tryptophan residue at position 115 chemically blocked by reaction with HNB-Br), 1–115, tyr, 116–169, tyr. The appendix tyr indicates that this peptide has been treated with a high excess of an oxidizing agent (the bromide adduct of 2-(2-nitrophenylsulphenyl)-3-methylindole) and its tyrosine residues have thus been oxidized. Peptide 1–36 (not previously described) was isolated directly from an acid extract of bovine brain tissue in a way analogous to the preparation of peptide 1–42 (cf. *Bergstrand* 1971) and characterized by the previously described techniques (cf. *Bergstrand* 1973). Equimolar concentrations were always used to compare the effect of the various peptides in the two *in vitro* tests.

TABLE 1. *Migration-inhibition Experiments Using Peritoneal Cells from 15 Animals Immunized with 25 μg 43–88 in FCA. Figures Give Migration Area in Arbitrary Units*

Animal No.	Control	43–88§	1–169	HNB 1–169	1–115, tyr	1–42	92–169	116–169, tyr	1–42 + 43–88	43–88 + 92–169
327	162	92	86	88	78	150	99	98	116	90
328	170	161	161	128	–	160	148	–	149	146
329	194	141	154	–	143	200	178	171	175	166
330	207	218	207	201	179	174	194	201	212	164
331	177	174	166	167	186	150	184	189	186	178
333	277	134	128	139	142	207	157	140	159	119
334	199	152	160	170	161	171	185	176	184	142
335	181	152	152	155	179	179	167	147	160	151
336	164	133	126	147	139	153	139	148	124	132
337	328	303	318	311	276	310	367	265	272	286
338	177	201	144	123	140	148	152	147	150	192
339	174	177	163	163	157	158	157	175	146	170
340	142	70	91	81	80	112	93	71	81	36
342	159	130	133	120	121	118	107	131	108	113
343	111	75	76	80	73	99	89	90	80	78
n			15	14	14	15	15	14	15	15
A†			0.92	0.88	0.92	0.37	0.70	0.86	0.77	1.10
s_A			0.0877	0.1277	0.1192	0.0995	0.1221	0.1077	0.1095	0.0990
$t_{A \neq 0}$			0.67	1.04	0.73	5.56***	2.69*(*)	1.20	2.03	-0.88
$t_{A \neq 1}$			8.20***	7.83***	8.13***	3.31**	6.17***	7.66***	6.83***	9.74***

§ Concentration of antigen in migration chamber 5nM = equimolar to 27 μg 43–88. Each antigen tested in two chambers, each containing two capillaries.

† For explanation of symbols A and s_A, see text.

Significance levels: * $0.01<p<0.05$
*(*) $0.01<p<0.02$
** $0.001<p<0.01$
*** $p<0.001$.

Statistics

The efficiency of different peptides to inhibit macrophage migration or to stimulate lymphocytes was evaluated by a multiple regression method earlier described and discussed in detail (cf. *Bergstrand* & *Källén* 1973c, d). Briefly, values registered without antigen (= controls; X_1), with antigen No. 1 (X_2), and with antigen No. 2 (Y) were compared using the equation:

$$\hat{Y} = A \cdot X_2 + (1-A) \cdot X_1$$

where A expresses the efficiency of antigen No. 2 as a fraction of that of antigen No. 1. If, for instance, the animals are immunized with BEP and tests performed with BEP (= antigen No. 1) and peptide 1–42 (= antigen No. 2), and if the A value of 1–42 is estimated to be 0.75, it means that peptide 1–42 can inhibit (in the MMI) or stimulate (in the LT) the cells to a degree corresponding to 75 per cent of that obtained by an equimolar amount of BEP*.

RESULTS

Macrophage Migration Inhibition Experiments

None of the tested peptides had any effect on the migration of peritoneal cells from guinea-pigs sensitized with saline in FCA (results not shown here), except for peptide 1–42 which was found to have a stimulating effect on such cells at high concentrations (10nM), as shown by *Bergstrand* & *Källén* 1973e).

Table 1 shows the results obtained by the MMI test on peritoneal cells from guinea-pigs sensitized with 25 μg of peptide 43–88 in FCA. For the convenience of readers not accustomed to the regression method of evaluation of the migration inhibition data, the actual migrated areas recorded are given *in extenso*. This Table clearly shows that all peptides examined were strongly inhibitory except peptide 1–42 which showed a moderate migration inhibiting effect.

* This way of expressing the relation between the registered variates differs slightly from our original usage; we previously used an *a*-value instead. The relation is A = (1-*a*), cf. *Bergstrand* & *Källén* (1973d).

Lymphoid Cell Transformation Experiments

In the LT test using rabbit lymph node cells, no effect of any of the peptides has been observed at the concentrations used when cells from rabbits immunized with saline in FCA were studied (*Bergstrand* & *Källén* 1973b).

Table 2 presents the data obtained by the LT test performed on cells from rabbits sensitized with one of the three main regions of the protein: peptides 1–42, 43–88, and

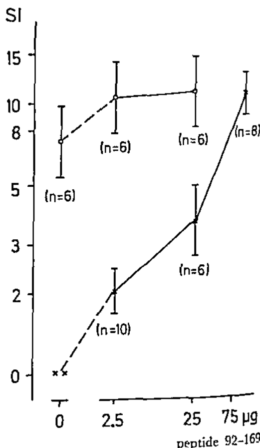


Fig. 1. Diagram showing stimulation index: SI = mean c.p.m. in antigen-stimulated cultures/mean c.p.m. in control cultures when different amounts of peptide 92–169 (expressed as μg given per rabbit) were used at immunization. SI are given on a log-scale. Means with standard error of the mean given for each group of rabbits, × = rabbits injected with peptide 92–169 only, o = rabbits injected with 150 μg of peptide 43–88, in some experiments mixed with known amounts of peptide 92–169, as shown on the abscissa.

TABLE 2. *"Cross-reactivity" between Main Regions of BEP in LT Tests*

Peptide used at immunization	Peptide used at test	No. of exper.	$A \pm s_A$	$t_{A \neq 0}$	$t_{A \neq 1}$
1–42	43–115, tyr	9	0.842 ± 0.086	9.8***	1.8
1–42	116–169, tyr	9	0.771 ± 0.086	9.0***	2.7*
43–88	1–42	6	0.671 ± 0.084	8.0***	3.9*
43–88	92–169	6	0.906 ± 0.084	10.8***	1.1
43–88	1–169	6	1.051 ± 0.125	8.4***	−0.4
92–169	1–36	6	0.662 ± 0.063	10.5***	5.4**
92–169	43–88	6	0.898 ± 0.063	14.3***	1.6
92–169	43–67	6	0.289 ± 0.063	4.6**	11.3***
92–169	68–88	6	−0.054 ± 0.080	−0.7	13.2***

The mode of estimate of $A \pm s_A$ *described in the text.* t-tests are performed for hypothesis A = O, i.e. tested peptide has no stimulating effect, and A = 1, i.e. tested peptide equals peptide used at immunization as stimulant. All peptides tested at concentrations equimolar with 50 μg/ml of BEP, i.e. 2.8 nM. *The mean stimulation index obtained is approximately 15 when peptide 1–42 is used for immunization and as stimulator, 9 when peptide 43–88 is used, and 12 when peptide 92–169 is used. Uptake in control cultures varied between 100 to 1000 c.p.m., depending on culture condition and source of lymph node.*

* $0.05 > p > 0.01$.
** $0.01 > p > 0.001$.
*** $p < 0.001$.

92–169. Each tested peptide is compared with the peptide used at immunization for capacity to stimulate the cells, using the A values described above. The Table shows that there is a high degree of stimulation when peptides 1–42 and 43–88 are tested on cells from animals injected with peptide 92–169. Analogous results are obtained when the two other peptides are used for immunization.

Previous investigations of the cross-reactivity between regions 43–88 and 92–169 in the MMI test indicate that at least two determinants in each region contribute to the effect (*Bergstrand & Källén* 1973a). In the LT tests shown in Table 2, a reactivity with peptide 43–67—but not with peptide 68–88—was found on cells from rabbits sensitized with peptide 92–169.

Is Peptide Contamination Responsible for the Cross-reactivity?

Previous studies indicate that peptide contamination at the level of the *in vitro* test cannot explain the cross-reactivity (*Bergstrand & Källén* 1973a). Figure 1 illustrates analyses of a series of experiments performed in order to investigate the importance of peptide contamination in the immunization of rabbits used in the LT tests. Animals were immunized by increasing amounts of peptide 92–169 (range 2.5 to 75 μg) and their lymph node cells were stimulated by this peptide at 2.8 nM (= 24 μg/ml). The stimulation index was found to increase with the amount of peptide 92–169 used for immunization. Cells from animals injected with a fixed amount (150 μg) of peptide 43–88 alone or mixed with various amounts of peptide 92–189 (as indicated in the figure) were stimulated by peptide 92–169 (24 μg/ml) to almost the same degree, irrespective of the *intentional* admixture of peptide 92–169.

If the stimulation of cells from rabbits immunized with peptide 43–88 and obtained by peptide 92–169 is to be explained exclusively as a result of contamination of the latter peptide with the former, it appears from the graph that more than 25 μg of peptide 92–169 in 150 μg of total peptide material is required—an amount far beyond that to be

easily detected by the analytical methods used (*Bergstrand* 1971, 1973).

DISCUSSION

The results presented in this report support the previous observations that different regions of the bovine encephalitogenic protein can apparently replace each other with high efficiency in the two *in vitro* tests used, MMI and LT (*Bergstrand* & *Källén* 1973a and 1973d).

These observations agree with and support the results obtained by *Hashim* & *Schilling* (1973) and *Hashim et al.* (1973). Using synthetic and natural peptides derived from the encephalitogenic protein, they showed that peptides 1–4 (5), 68–73, and 116–121 completely cross-reacted in delayed-type skin reaction tests, apparently irrespective of the possible presence of an extra serine residue in positions 2 (cf. *Brostoff et al.* 1974).

The present report registers strong "cross-reactivity" effects; only that of peptide 1–42 on cells sensitized with peptide 43–88 and tested in MMI is moderate. The interpretation of the latter observation is further complicated by the phenomenon previously described *Bergstrand* & *Källén* (1973e), namely that peptide 1–42 at high concentrations (10 nM) can stimulate the migration of peritoneal cells from animals sensitized with FCA and saline; it is not known whether inhibiting and enhancing factors are working simultaneously in this system.

Cumulating data indicate that there is a high degree of cross reactivity between different parts of BEP. In one way or another, peptides 1–4 (or 1–19), 43–67, 68–88, 116–121, 133–149, and 153–169 have all been implicated in cross-reactions (cf. *Hashim* & *Schilling* 1973, *Hashim et al.* 1973, *Bergstrand* & *Kallén* 1973a, and present work).

What is the Basis of the Cross-reactivity?

Various explanations of the phenomenon that different parts of the BEP protein can replace each other in the tests described can be suggested (cf. *Bergstrand* & *Källén* 1973a):

a) Contamination of the immunizing peptide with material from the "cross-reactive" peptide.

b) A cross-reactivity based on structural similarities of different antigenic determinants in the molecule.

c) A cross-reactivity on the cell level, i.e. some kind of multipotency of antigen-reactive cells.

Evidence previously obtained (*Bergstrand* & *Källén* 1973a) is in favour of explanations b), and c) and the present results seem to be in support of this. Alternative a) seems unlikely for the following reasons: If contamination is the only explanation of the replacement phenomenon (alternative a) and if the effect of the contaminating peptide is low, then the intact protein should have an effect close to that seen in case of the immunizing peptide. If, on the other hand, the contaminating peptide induces a marked reaction, then the intact protein should exert an effect markedly stronger than that to be obtained by the peptide used for immunization. Keeping this in mind, we performed MMI tests (Table 1) where peptide 43–88 was used as immunizing agent. Both peptides 1–42 and 92–169 had moderate to strong effects (A = 0.37 and 0.60, resp.); yet, the intact protein, 1–170, or mixtures of peptide 43–88 and 1–42 or 92–169, did not inhibit the cell migration significantly more than did peptide 43-88. Similarly, in the LT test (Table 2) after immunization with peptide 43–88, peptides 1–42 and 92–169 both gave marked responses (A = 0.67 and 0.91, resp.), but the intact protein gave only slightly and not significantly higher stimulation than peptide 43–88 (A = 1.05).

Furthermore, we performed LT tests with a view to examining the degree of contamination of the immunizing peptide required to induce an immune response to the contaminating peptide. It was found that if a degree of stimulation similar to that observed in the LT tests was to be obtained, contamination

should amount to more than 15 per cent. This is excluded by the purity criteria used. Thus, the data apparently do not fit the "contamination" alternative.

Alternative c) is attractive in the light of recent observations by *DeLuca et al.* (1974) indicating the existence of multipotent antigen binding cells. If cells from animals sensitized to the whole protein are tested with the three main peptides (1–42, 43–88, 92–169), peptide 43–88 is apparently the one least capable of reacting both in the MMI and in the LT test (*Bergstrand* & *Källén* 1974). If explanation c) were true, peptides 1–42 and 92–169 could be expected to be more effective than peptide 43–88 also when cells from animals immunized with peptide 43–88 are tested. This is not the case (Table 1 and 2). However, if a clone of cells in the non-immune animal reacts to the intact BEP or any of its peptide parts, this clone can be stimulated at the immunization with one peptide, e.g., peptide 43–88, in order to give cells with increased amount of receptors specific for the immunizing peptide or with increased receptor avidity, but with receptors for other determinants on the protein retained. This dynamic form of alternative c) would fit the experimental data.

The remaining alternative explanation, b), supposes that a true structural cross-reactivity between different regions of the protein occurs, i.e. they can more or less efficiently compete on the receptor level. Thus, the peptide used for immunization would always be the most efficient stimulator, as was also found to be the case. The recent observation by *Alvord et al.* (1974) that at least eight regions, uniformly spread throughout the entire BEP molecule, show sequence similarities favours the last-mentioned alternative explanation.

The costs of this investigation were defrayed by grants from the *Swedish Medical Research Foundation* (project 12X-3920) and from the *Research Funds of the Medical Faculty*

REFERENCES

Alvord, E. C., Jr., Hruby, S., Petersen, R., Barker, W. C. & Dayhoff, M. O.: Antigenic analysis of fragments of myelin membrane encephalitogenic protein. Fed. Proc. 33 Abstract 3268, 1974.

Bergstrand, H.: Isolation and partial characterization of some proteolytically and chemically derived fragments of bovine encephalitogenic protein. Eur. J. Biochem. *21:* 116–124, 1971.

Bergstrand, H.: Localization of antigenic determinants on bovine encephalitogenic protein. Further studies with the macrophage migration inhibition assay in guinea pigs. Immunochemistry *10:* 611–620, 1973.

Bergstrand, H. & Källén, B.: Localization of antigenic determinants on bovine encephalitogenic protein. Studies in guinea pigs with macrophage migration inhibition. Cell. Immunol. *3:* 660–671, 1972.

Bergstrand, H. & Källén, B.: Is there a cross-reactivity between different parts of the bovine encephalitogenic protein in the macrophage migration inhibition assay? Immunochemistry *10:* 471–476, 1973a.

Bergstrand, H. & Källén, B.: Antigenic determinants on bovine encephalitogenic protein. Localization of regions that induce transformation of lymph node cells from immunized rabbits. Eur. J. Immunol. *3:* 287–292, 1973b.

Bergstrand, H. & Källén, B.: On the statistical evaluation of the macrophage migration inhibition assay. Scand. J. Immunol. *2:* 173–187, 1973c.

Bergstrand, H. & Källén, B.: Antigenic determinant on bovine encephalitogenic protein: Studies in rabbits with derivatives of fragment 1–43 and the lymph node cell transformation test. Neurobiology *3:* 246–255, 1973d.

Bergstrand, H. & Källén, B.: Antigenic determinants in the N-terminal part of bovine encephalitogenic protein studied with the macrophage migration inhibition assay in guinea pigs. Evidence for an immunological reactivity of peptide 1–43 when tested on cells from animals injected with Freund's complete adjuvant. Immunochemistry *10:* 229–238, 1973e.

Bergstrand, H. & Källén, B.: Further studies on the antigenic regions of bovine encephalitogenic protein with the lymph node cell transformation test in rabbits. Neurobiology *4:* 328–336, 1974.

Brostoff, S. W., Reuter, W., Hichens, M. & Eylar, E. H.: Specific cleavage of the A1 protein from myelin with cathepsin D. J. Biol. Chem. *249:* 559–567, 1974.

De Luca, D., Decker, J., Miller, A & Sercarz, E.: Antigen binding to lymphoid cells from unim-

munized mice: High frequency of beta-galactosidase binding cells at optimal conditions. Cell. Immunol. *10:* 1–18, 1974.

Hashim, G. A., Hwang, F. & Schilling, F. J.: Experimental allergic encephalomyelitis: Basic protein regions responsible for delayed type hypersensitivity. Arch. Biochem. Biophys. *156:* 298–309, 1973.

Hashim, G. A. & Schilling, F. J.: Allergic encephalomyelitis: Characterization of the determinants for delayed type hypersensitivity. Biochem. Biophys. Res. Commun. *50:* 589–596, 1973.

Rajewsky, K. & Mohr, R.: Specificity and heterogeneity of helper T cells in the response to serum albumins in mice. Eur. J. Immunol. *4:* 111–119, 1974.

Weinbaum, F. I., Butchko, G. M., Lerman, S., Thorbecke, G. J. & Nisonoff, A.: Comparison of cross-reactivities between albumins of various species at the level of antibody and helper T cells—studies in mice. J. Immunol. *113:* 257–265, 1974.

Acta path. microbiol. scand. Sect. C, *83:* 173–176, 1975

HUMAN IgM INTERACTING WITH STAPHYLOCOCCAL PROTEIN A

ARNE GROV

The University of Bergen, School of Medicine, The Gade Institute, Department of Microbiology, Bergen, Norway

Grov, A. Human IgM interacting with staphylococcal protein A. Acta path. microbiol. scand. Sect. C, *83:* 173–176, 1975.

IgM from 7 sera of patients with macroglobulinemia has been examined for interaction with staphylococcal protein A. Three of the IgM proteins were fixed to a protein A-Sepharose column. They gave a direct co-precipitation and the "star-phenomenon", but no direct precipitation in agar. The primary protein A reactive sites were localized to the Fc-region, but co-precipitation and "star"-formation were also dependent on other molecular configurations. Two of the sera examined contained both protein A reactive and protein A non-reactive IgM in different ratio.

Key words: IgM; staphylococcal protein A.

A. Grov, The University of Bergen, School of Medicine, The Gade Institute, Department of Microbiology, Bergen, Norway.

Received 29.xi.74 Accepted 29.xi.74

Protein A of *Staphylococcus aureus* interacts with mammalian immunoglobulins of the IgG class owing to its affinity for the Fc-region (2, 9, 8, 4). Some myeloma IgG proteins do not form precipitate with protein A, but are able to inhibit precipitation between protein A and a precipitating IgG (9), to form soluble complexes with protein A, and to participate in a direct co-precipitation and the "star phenomenon" (7, 10). Other immunoglobulin classes have been supposed to be unreactive in this context (9, 8). However, *McDowell et al.* (13) have shown that protein A precipitates with IgA of normal human colostrum and also with IgM from rabbits and guinea pigs immunized with protein A, although the mechanisms of these interactions were not studied.

While working with an IgM protein from a patient with Waldenström's macroglobulinemia (serum As), evidence was obtained which suggested that this IgM interacts with protein A. This paper presents the results of a closer examination of the observation.

MATERIALS AND METHODS

Immunoglobulin Preparations

IgM_{As} was isolated from serum As (containing 254 mg of IgM (λ)/ml) by gel filtration on a column of Sephadex G-200 (2.3 × 90 cm), stabilized and eluted with phosphate-buffered saline (PBS) containing 0.03 M NaN_3. The fractions at the ascending part of the macroglobulin peak were pooled, concentrated by ultrafiltration (Amicon Corp., Mass., USA) and refiltered on the same column of Sephadex. Pooled fractions were then concentrated to approximately 10 mg protein/ml as determined according to the description in (12), and checked for purity by immunoelectrophoresis against specific antisera.

IgM proteins were similarly isolated from six other macroglobulinemia sera, randomly selected from the routine laboratory. Two of these sera contained biclonal IgM (κ), the other four were monoclonal IgM (κ) proteins.

IgG was prepared from pools of normal human and normal rabbit sera by precipitation in 50 per cent $(NH_4)_2SO_4$ after prior batch adsorption with DEAE-Sephadex A-50 (Pharmacia, Sweden), and was further purified on columns of DEAE-cellulose (Eastman, N.Y., USA) according to the method of *Fahey & McLaughlin* (1).

Subunits of IgM_{As}. 7S subunits with reduced and alkylated interchain disulphide bonds, and 7S subunits with intact interchain disulphide bonds were prepared as described by *Harboe & Solheim* (6) and *Solheim* (15).

Fabμ and $(Fc)_5\mu$ fragments. IgM was digested with bovine trypsin (Type I, Sigma Chemical Co, Mo., USA) at 56° C for 30 min in 0.05 M Tris-HCl buffer, pH 8.0, containing 0.01 M $CaCl_2$, with an enzyme to protein ratio of 1:25 (wt/wt). The reaction was stopped by the addition of trypsin soybean inhibitor (Type I-S, Sigma) and the resulting fragments were separated by gel filtration on Sephadex G-200 (14).

Monomeric Fcμ fragments were prepared by reduction of isolated $(Fc)_5\mu$ fragments in 0.2 M 2-mercaptoethanol and alkylation with iodoacetamide (6).

Antisera

Antisera specific to μ, α and γ heavy chains and to λ and κ light chains were obtained from Hyland, Belgium.

Antigen

Protein A was prepared as described in (3).

Immunosorbent

Columns of protein A coupled to Sepharose 4B (Pharmacia) were prepared and used as previously (4).

Immunoelectrophoresis

Immunoelectrophoretic analyses were performed using an LKB apparatus, 1 per cent agar Noble (Difco, Mich, USA) in barbitone buffer (I 0 025, pH 8.6), and a voltage of 5 to 6 V/cm.

Double Diffusion in Agar, Inhibition of Precipitation (4), and Tests for Co-precipitations and the "Star Phenomenon" (7, 10, 11)

were carried out in 1 per cent special agar Noble in barbitone buffer (I 0 025, pH 8.6) or in PBS.

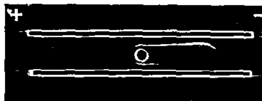


Fig. 1. Immunoelectrophoresis of IgM_{As} (10 mg/ml) in barbitone buffer, pH 8 6, I 0.025, for 2 h at 5 V/cm. Anti-μ serum in the upper and anti-λ in the lower trough.

RESULTS

The isolated IgM_{As} protein was found to react with anti-μ and anti-λ sera (Fig. 1). No reaction with any of the other sera was observed. When applied to the protein A-Sepharose column the purified IgM_{As} was completely fixed and was then eluted with 3 M NaSCN. After extensive dialysis of the eluate in PBS, and concentration by ultrafiltration, all the protein was found to be re-fixed to the protein A-Sepharose column. The eluted protein (concentrated to about 10 mg/ml) showed a pure IgM line on immunoelectrophoresis. It did not precipitate with protein A on double diffusion in agar gel or inhibit the reaction between protein A and normal human IgG, but gave co-precipitation with normal rabbit IgG and the "star phenomenon" (Fig. 2), the third component being either normal human or normal rabbit IgG, unlike the usual set-up (10, 11). The

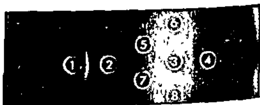


Fig. 2. Double diffusion in agar Wells: (1) IgM_{As} (10 mg/ml) + normal rabbit IgG (10 mg/ml) (1 1), (2) and (3) protein A, 0.5 mg/ml; (4) and (6) normal rabbit IgG, 10 mg/ml; (5) and (7) IgM_{As}, 10 mg/ml; (8) normal human IgG, 10 mg/ml. A direct co-precipitate is formed between (1) and (2) and a "star" between the three-component system (3)-(5)-(6) as well as between (3)-(7)-(8). Note there is no precipitate between protein A (2) and IgM_{As} (5) and (7) or between protein A (3) and normal rabbit IgG (4).

line formed in the inhibition system was not confluent with that between protein A and normal human IgG and is most probably also due to a co-precipitation mechanism.

Reduction of IgM_{As} by 2-mercaptoethanol or cysteine followed by alkylation did not alter the ability to combine with protein A as demonstrated both by the inhibition test and by binding to the protein A-Sepharose column. In contrast, the ability to co-precipitate and to form a "star" was lost. Similarly, trypsinated IgM_{As} neither gave co-precipitation nor "star"-formation. One part of the trypsinated protein was bound to the protein A column, the other was inactive in this context. Fractionation of a trypsin digest by gel filtration revealed that the protein A reactive fraction corresponded to the $(Fc)_5\mu$ and the protein A non-reactive fraction to the $Fab\mu$ fragments. Further reduction of the $(Fc)_5\mu$-fragments by 2-mercaptoethanol and alkylation of the monomeric $Fc\mu$-fragments formed did not alter the interaction with protein A. The fragments were fixed to the protein A column, but were inactive in the co-precipitation tests.

Two of the other 6 isolated IgM proteins also interacted with protein A. Both preparations produced the "star" and co-precipitated with normal rabbit IgG. Testing on the protein A column revealed the presence in both preparations of material non-reactive to protein A, approximately one third and two thirds of the total IgM, respectively.

Neither "star"-formation nor co-precipitations were observed with the 4 purified IgM proteins which did not fix to the protein A column.

DISCUSSION

It is evident that IgM proteins of some sera from patients with macroglobulinemia have sites for interaction with staphylococcal protein A. However, none of the protein A reactive IgM proteins which were studied precipitated with protein A in double diffusion as most human IgG globulins do. The interactions were similar to that of inhibiting myeloma globulins in that they formed a "star" with precipitating IgG (7, 11). Furthermore, the IgM proteins also behaved like $F(ab')_2$ from sera precipitating protein A in that they produced a direct co-precipitate as well as a "star" with normal rabbit IgG.

In a recent study by *Harboe* & *Følling* (5) two distinct groups of human IgM were revealed, both based on binding to staphylococci. However, it was shown that the binding site of the staphylococci was not protein A, the interaction being rather labile and dependent on the tertiary configuration of the IgM molecule.

As in normal human IgG, the primary protein A reactive sites of the IgM proteins in the present study apparently reside in the Fc-region. Both penta- and monomeric Fc-fragments of IgM_{As}, in contrast to Fab-fragments, were fixed to the protein A-Sepharose column. It has been suggested that precipitation of normal IgG with protein A depends on molecular configurations which differ from primary protein A reacting structures (7), and studies on precipitating myeloma globulins and on the "star phenomenon" have indicated additional mechanisms of γ-globulin interactions. The ability of the IgM proteins to co-precipitate and to form a "star" apparently also depends on other configurations than the primary protein A reactive sites. While splitting of non-covalent bonds by exposure of the IgM to NaSCN did not alter the reactivity pattern, reduction and alkylation of disulphide bonds destroyed the co-precipitating and "star"-forming ability, but not the ability to bind protein A.

Of special interest is the observation that two of the sera contained both protein A reactive and protein A non-reactive IgM, and that their ratio varied. It is thus tempting to suggest that protein A reactive IgM may be present in all human sera, but in variable amounts, and probably also in sera of other species with quantitative variations as for protein A-reacting IgG (7, 4).

IgM proteins were similarly isolated from six other macroglobulinemia sera, randomly selected from the routine laboratory. Two of these sera contained biclonal IgM (κ), the other four were monoclonal IgM (κ) proteins.

IgG was prepared from pools of normal human and normal rabbit sera by precipitation in 50 per cent $(NH_4)_2SO_4$ after prior batch adsorption with DEAE-Sephadex A-50 (Pharmacia, Sweden), and was further purified on columns of DEAE-cellulose (Eastman, N.Y., USA) according to the method of *Fahey & McLaughlin* (1).

Subunits of IgM_{As}. 7S subunits with reduced and alkylated interchain disulphide bonds, and 7S subunits with intact interchain disulphide bonds were prepared as described by *Harboe & Solheim* (6) and *Solheim* (15).

Fabμ and $(Fc)_5μ$ fragments. IgM was digested with bovine trypsin (Type I, Sigma Chemical Co., Mo., USA) at 56° C for 30 min in 0.05 M Tris-HCl buffer, pH 8.0, containing 0.01 M $CaCl_2$, with an enzyme to protein ratio of 1:25 (wt/wt). The reaction was stopped by the addition of trypsin soybean inhibitor (Type I-S, Sigma) and the resulting fragments were separated by gel filtration on Sephadex G-200 (14).

Monomeric Fcμ fragments were prepared by reduction of isolated $(Fc)_5μ$ fragments in 0.2 M 2-mercaptoethanol and alkylation with iodoacetamide (6).

Antisera

Antisera specific to μ, α and γ heavy chains and to λ and κ light chains were obtained from Hyland, Belgium.

Antigen

Protein A was prepared as described in (3).

Immunosorbent

Columns of protein A coupled to Sepharose 4B (Pharmacia) were prepared and used as previously (4).

Immunoelectrophoresis

Immunoelectrophoretic analyses were performed using an LKB apparatus, 1 per cent agar Noble (Difco, Mich., USA) in barbitone buffer (I 0.025, pH 8.6), and a voltage of 5 to 6 V/cm.

Double Diffusion in Agar, Inhibition of Precipitation (4), and Tests for Co-precipitations and the "Star Phenomenon" (7, 10, 11)

were carried out in 1 per cent special agar Noble in barbitone buffer (I 0.025, pH 8.6) or in PBS.

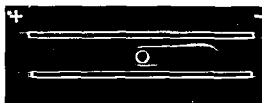


Fig. 1. Immunoelectrophoresis of IgM_{As} (10 mg/ml) in barbitone buffer, pH 8.6, I 0.025, for 2 h at 5 V/cm. Anti-μ serum in the upper and anti-λ in the lower trough.

RESULTS

The isolated IgM_{As} protein was found to react with anti-μ and anti-λ sera (Fig. 1). No reaction with any of the other sera was observed. When applied to the protein A-Sepharose column the purified IgM_{As} was completely fixed and was then eluted with 3 M NaSCN. After extensive dialysis of the eluate in PBS, and concentration by ultrafiltration, all the protein was found to be re-fixed to the protein A-Sepharose column. The eluted protein (concentrated to about 10 mg/ml) showed a pure IgM line on immunoelectrophoresis. It did not precipitate with protein A on double diffusion in agar gel or inhibit the reaction between protein A and normal human IgG, but gave co-precipitation with normal rabbit IgG and the "star phenomenon" (Fig. 2), the third component being either normal human or normal rabbit IgG, unlike the usual set-up (*10, 11*). The

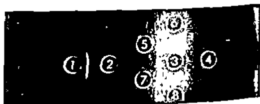


Fig 2. Double diffusion in agar. Wells: (1) IgM_{As} (10 mg/ml) + normal rabbit IgG (10 mg/ml) (1:1); (2) and (3) protein A, 0.5 mg/ml; (4) and (6) normal rabbit IgG, 10 mg/ml; (5) and (7) IgM_{As}, 10 mg/ml; (8) normal human IgG, 10 mg/ml. A direct co-precipitate is formed between (1) and (2) and a "star" between the three-component system (3)-(5)-(6) as well as between (3)-(7)-(8). Note there is no precipitate between protein A (2) and IgM_{As} (5) and (7) or between protein A (3) and normal rabbit IgG (4).

line formed in the inhibition system was not confluent with that between protein A and normal human IgG and is most probably also due to a co-precipitation mechanism.

Reduction of IgM_{A*} by 2-mercaptoethanol or cysteine followed by alkylation did not alter the ability to combine with protein A as demonstrated both by the inhibition test and by binding to the protein A-Sepharose column. In contrast, the ability to co-precipitate and to form a "star" was lost. Similarly, trypsinated IgM_{A*} neither gave co-precipitation nor "star"-formation. One part of the trypsinated protein was bound to the protein A column, the other was inactive in this context. Fractionation of a trypsin digest by gel filtration revealed that the protein A reactive fraction corresponded to the $(Fc)_5\mu$ and the protein A non-reactive fraction to the Fabμ fragments. Further reduction of the $(Fc)_5\mu$-fragments by 2-mercaptoethanol and alkylation of the monomeric Fcμ-fragments formed did not alter the interaction with protein A. The fragments were fixed to the protein A column, but were inactive in the co-precipitation tests.

Two of the other 6 isolated IgM proteins also interacted with protein A. Both preparations produced the "star" and co-precipitated with normal rabbit IgG. Testing on the protein A column revealed the presence in both preparations of material non-reactive to protein A, approximately one third and two thirds of the total IgM, respectively.

Neither "star"-formation nor co-precipitations were observed with the 4 purified IgM proteins which did not fix to the protein A column.

DISCUSSION

It is evident that IgM proteins of some sera from patients with macroglobulinemia have sites for interaction with staphylococcal protein A. However, none of the protein A reactive IgM proteins which were studied precipitated with protein A in double diffusion as most human IgG globulins do. The interactions were similar to that of inhibiting myeloma globulins in that they formed a "star" with precipitating IgG (7, 11). Furthermore, the IgM proteins also behaved like $F(ab')_2$ from sera precipitating protein A in that they produced a direct co-precipitate as well as a "star" with normal rabbit IgG.

In a recent study by *Harboe & Fölling* (5) two distinct groups of human IgM were revealed, both based on binding to staphylococci. However, it was shown that the binding site of the staphylococci was not protein A, the interaction being rather labile and dependent on the tertiary configuration of the IgM molecule.

As in normal human IgG, the primary protein A reactive sites of the IgM proteins in the present study apparently reside in the Fc-region. Both penta- and monomeric Fc-fragments of IgM_{A*}, in contrast to Fab-fragments, were fixed to the protein A-Sepharose column. It has been suggested that precipitation of normal IgG with protein A depends on molecular configurations which differ from primary protein A reacting structures (7), and studies on precipitating myeloma globulins and on the "star phenomenon" have indicated additional mechanisms of γ-globulin interactions. The ability of the IgM proteins to co-precipitate and to form a "star" apparently also depends on other configurations than the primary protein A reactive sites. While splitting of non-covalent bonds by exposure of the IgM to NaSCN did not alter the reactivity pattern, reduction and alkylation of disulphide bonds destroyed the co-precipitating and "star"-forming ability, but not the ability to bind protein A.

Of special interest is the observation that two of the sera contained both protein A reactive and protein A non-reactive IgM, and that their ratio varied. It is thus tempting to suggest that protein A reactive IgM may be present in all human sera, but in variable amounts, and probably also in sera of other species with quantitative variations as for protein A-reacting IgG (7, 4).

REFERENCES

1. *Fahey, J. L. & McLaughlin, C.:* Preparation of antisera specific for 6.6 S γ-globulins, β_{2A}-globulins, γ_1-macroglobulins, and for type I and II common γ-globulin determinants. J. Immunol. *91:* 484–497, 1963.
2. *Forsgren, A. & Sjöquist, J.:* Protein A from *S. aureus.* I. Pseudoimmune reaction with human γ-globulin. J. Immunol. *97:* 822–827, 1966.
3. *Grov, A.:* Studies on antigen preparations from *Staphylococcus aureus.* 4. Separation and purification of protein A and a related precipitinogen. Acta path. microbiol. scand. *73:* 400–406, 1968.
4. *Grov, A.:* Studies on the interaction between staphylococcal protein A and the Fc-region of immunoglobulin G. Acta path. microbiol. scand. Sect. A, Suppl. *236:* 77–83, 1973.
5. *Harboe, M. & Fölling, I.:* Recognition of two distinct groups of human IgM and IgA based on different binding to staphylococci. Scand. J. Immunol. *3:* 471–482, 1974.
6. *Harboe, M. & Solheim, B. G.:* Formation of hybrid IgM subunits. Scand. J. Immunol. *1:* 63–73, 1972.
7. *Kronvall, G.:* Interactions between staphylococcal protein A and γ-globulins. Ph.D. Thesis, University of Lund, Sweden, 1971.
8. *Kronvall, G., Seal, U. S., Finstad, J. & Williams, R. C., Jr.:* Phylogenetic insight into evolution of mammalian Fc fragment of γG globulin using staphylococcal protein A. J. Immunol. *104:* 140–147, 1970.
9. *Kronvall, G. & Williams, R. C., Jr.:* Differences in anti-protein A activity among IgG subgroups. J. Immunol. *103:* 828–833, 1969.
10. *Kronvall, G. & Williams, R. C., Jr.:* The star phenomenon, a three component immunoprecipitation involving protein A. Immunochemistry *8:* 577–580, 1971.
11. *Lind, I. & Mansa, B.:* Immunochemical study of the interaction between staphylococcal protein A, rabbit antistaphylococcal sera, and selected sera from non-immunized animals. Scand. J. Immunol. *3:* 147–156, 1974.
12. *Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. L.:* Protein measurement with Folin phenol reagent. J. biol. Chem. *193:* 265–275, 1951.
13. *McDowell, G., Grov, A. & Oeding, P.:* IgA and IgM reacting with staphylococcal protein A. Acta path. microbiol. scand. Sect. B, *79:* 801–804, 1971.
14. *Plaut, A. G. & Tomasi, T. B., Jr.:* Immunoglobulin M: Pentameric Fcμ fragments released by trypsin at higher temperatures. Proc. nat. Acad. Sci. (Wash.) *65:* 318–322, 1970.
15. *Solheim, B. G.:* Reassociation of reduced IgM to covalently bound 8S and 17S molecules. *Acta path. microbiol. scand. Sect.* B, *80:* 769–772, 1972.

Acta path. microbiol. scand. Sect. C, 83: 177–183, 1975

IMMUNE DEFICIENCY IN SEX-LINKED HEREDITARY THROMBOCYTOPENIA

VAGN ANDERSEN, MOGENS THOMSEN and JØRGEN COHN

Laboratory of Clinical Immunology, Department of Medicine TA; Tissue-Typing Laboratory, Blood Bank, and Blood-Grouping Department; and Department of Paediatrics G, Rigshospitalet, University Hospital of Copenhagen, Denmark

Andersen, V., Thomsen, M. & Cohn, J. Immune deficiency in sex-linked hereditary thrombocytopenia. Acta path. microbiol. scand. Sect. C, 83: 177–183, 1975.

Eleven thrombocytopenic members of a large family with sex-linked hereditary thrombocytopenia were studied. Routine immunological investigation revealed little evidence of immune deficiency, but abnormal results were obtained in studies of the *in vitro* blast transformation response of the patients' lymphocytes. Impaired responses to microbial antigens were observed in all patients thus studied, whereas a decreased response to mitogens was observed in only one patient. Of the non-thrombocytopenic family members, approximately half showed subnormal responses to one microbial extract. These results, taken together with the increased incidence of infections in the thrombocytopenic patients, indicate the necessity for immunological scrutiny of patients with hereditary thrombocytopenia, in particular when splenectomy is considered.

Key words: Immune deficiency; lymphocyte transformation; hereditary thrombocytopenia; sex-linked.

V. Andersen, Department of Medicine TA, Rigshospitalet, Tagensvej 18, DK-2200 Copenhagen N, Denmark.

Received 14.x.74 Accepted 27.xi.74

The association of immune deficiencies with hereditary thrombocytopenia is best known in the Wiskott-Aldrich syndrome which is a sex-linked recessive disorder characterized by thrombocytopenia, eczema, and increased incidence and severity of infections; untreated, it runs an early fatal course, a major course of death being infection.

In sex-linked hereditary thrombocytopenia without other obvious features of the Wiskott-Aldrich syndrome immunological abnormalities have been reported (4, 15). However, in vitro-studies of lymphocyte function have been carried out in only one such patient (15) although important deficiencies have been found in the Wiskott-Aldrich syndrome (11). In this communication, the results of immunological studies of 11 members of a large family with sex-linked thrombocytopenia are reported. Impaired lymphocyte function was demonstrated, in accordance with the clinical experience of decreased resistance to infections in these patients.

SUBJECTS

The persons studied are members of a family with sex-linked recessive thrombocytopenia described by *Cohn et al.* (5). Table 1 summarizes the most pertinent clinical and laboratory findings in the 11 afflicted males studied, in 6 of these it was possible to carry out a detailed study of lympho-

REFERENCES

1. *Fahey, J. L.* & *McLaughlin, C.:* Preparation of antisera specific for 6.6 S γ-globulins, β_{2A}-globulins, γ_1-macroglobulins, and for type I and II common γ-globulin determinants. J. Immunol. *91:* 484–497, 1963.
2. *Forsgren, A.* & *Sjöquist, J.:* Protein A from *S. aureus.* I. Pseudoimmune reaction with human γ-globulin. J. Immunol. *97:* 822–827, 1966.
3. *Grov, A.:* Studies on antigen preparations from *Staphylococcus aureus.* 4. Separation and purification of protein A and a related precipitinogen. Acta path. microbiol. scand. *73:* 400–406, 1968.
4. *Grov, A.:* Studies on the interaction between staphylococcal protein A and the Fc-region of immunoglobulin G. Acta path. microbiol. scand. Sect. A, Suppl. *236:* 77–83, 1973.
5. *Harboe, M.* & *Fölling, I.:* Recognition of two distinct groups of human IgM and IgA based on different binding to staphylococci. Scand. J. Immunol. *3:* 471–482, 1974.
6. *Harboe, M.* & *Solheim, B. G.:* Formation of hybrid IgM subunits. Scand. J. Immunol. *1:* 63–73, 1972.
7. *Kronvall, G.:* Interactions between staphylococcal protein A and γ-globulins. Ph.D. Thesis, University of Lund, Sweden, 1971.
8. *Kronvall, G., Seal, U. S., Finstad, J.* & *Williams, R. C., Jr.:* Phylogenetic insight into evolution of mammalian Fc fragment of γG globulin using staphylococcal protein A. J. Immunol. *104:* 140–147, 1970.
9. *Kronvall, G.* & *Williams, R. C., Jr.:* Differences in anti-protein A activity among IgG subgroups. J. Immunol. *103:* 828–833, 1969
10. *Kronvall, G.* & *Williams, R. C., Jr.:* The star phenomenon, a three component immunoprecipitation involving protein A. Immunochemistry *8:* 577–580, 1971.
11. *Lind, I.* & *Mansa, B.:* Immunochemical study of the interaction between staphylococcal protein A, rabbit antistaphylococcal sera, and selected sera from non-immunized animals. Scand. J. Immunol. *3:* 147–156, 1974.
12. *Lowry, O. H., Rosebrough, N. J., Farr, A L.* & *Randall, R. L.:* Protein measurement with Folin phenol reagent. J. biol. Chem. *193:* 265–275, 1951.
13. *McDowell, G., Grov, A.* & *Oeding, P.:* IgA and IgM reacting with staphylococcal protein A. Acta path. microbiol. scand. Sect. B, *79:* 801–804, 1971.
14. *Plaut, A. G.* & *Tomasi, T. B., Jr.:* Immunoglobulin M: Pentameric Fcμ fragments released by trypsin at higher temperatures. Proc. nat. Acad. Sci. (Wash.) *65:* 318–322, 1970.
15. *Solheim, B. G.:* Reassociation of reduced IgM to covalently bound 8S and 17S molecules. *Acta path. microbiol. scand.* Sect. B, *80:* 769–772, 1972.

Acta path. microbiol. scand. Sect. C, 83: 177–183, 1975

IMMUNE DEFICIENCY IN SEX-LINKED HEREDITARY THROMBOCYTOPENIA

Vagn Andersen, Mogens Thomsen and Jorgen Cohn

Laboratory of Clinical Immunology, Department of Medicine TA; Tissue-Typing Laboratory, Blood Bank, and Blood-Grouping Department; and Department of Paediatrics G, Rigshospitalet, University Hospital of Copenhagen, Denmark

Andersen, V., Thomsen, M. & Cohn, J Immune deficiency in sex-linked hereditary thrombocytopenia. Acta path. microbiol. scand. Sect. C, 83: 177–183, 1975.

Eleven thrombocytopenic members of a large family with sex-linked hereditary thrombocytopenia were studied. Routine immunological investigation revealed little evidence of immune deficiency, but abnormal results were obtained in studies of the *in vitro* blast transformation response of the patients' lymphocytes. Impaired responses to microbial antigens were observed in all patients thus studied, whereas a decreased response to mitogens was observed in only one patient. Of the non-thrombocytopenic family members, approximately half showed subnormal responses to one microbial extract. These results, taken together with the increased incidence of infections in the thrombocytopenic patients, indicate the necessity for immunological scrutiny of patients with hereditary thrombocytopenia, in particular when splenectomy is considered.

Key words: Immune deficiency; lymphocyte transformation; hereditary thrombocytopenia; sex-linked.

V. Andersen, Department of Medicine TA, Rigshospitalet, Tagensvej 18, DK-2200 Copenhagen N, Denmark.

Received 14.x.74 Accepted 27.xi.74

The association of immune deficiencies with hereditary thrombocytopenia is best known in the Wiskott-Aldrich syndrome which is a sex-linked recessive disorder characterized by thrombocytopenia, eczema, and increased incidence and severity of infections; untreated, it runs an early fatal course, a major course of death being infection.

In sex-linked hereditary thrombocytopenia without other obvious features of the Wiskott-Aldrich syndrome immunological abnormalities have been reported (4, 15). However, *in vitro*-studies of lymphocyte function have been carried out in only one such patient (15) although important deficiencies have been found in the Wiskott-Aldrich syndrome (11). In this communication, the results of immunological studies of 11 members of a large family with sex-linked thrombocytopenia are reported. Impaired lymphocyte function was demonstrated, in accordance with the clinical experience of decreased resistance to infections in these patients.

SUBJECTS

The persons studied are members of a family with sex-linked recessive thrombocytopenia described by *Cohn et al.* (5). Table 1 summarizes the most pertinent clinical and laboratory findings in the 11 afflicted males studied; in 6 of these it was possible to carry out a detailed study of lympho-

cyte transformation responses *in vitro*. At the time of study, all were well, in particular without symptoms and signs of infection. None had received blood transfusions within the past 6 months. Besides, 45 relatives (siblings, parents, and grandparents) were examined; none of these had any increased incidence of infections, and their haematological status was normal.

Lymphocyte cultures from healthy control subjects within the same age range as the family members studied were run in parallel. These results are given in the figures and are identical to those obtained in 27 healthy adults, studied over a longer period in our laboratory.

METHODS

Lymphocyte Cultures

30 ml of blood were drawn in equal volumes of RPMI 1640 with heparin. The blood was shipped at room temperature. Lymphocytes + monocytes were isolated on a Ficoll-Isopaque gradient within 10 hours of sampling and incubated overnight in medium containing 15 per cent pooled A-serum from non-transfused young male donors. The cells were washed twice in RPMI 1640 with 5 per cent serum, and 10^5 cells per vial were cultured in 500 μl of RPMI with 15 per cent serum. All cultures were set up in triplicate.

Mitogens

Phytohaemagglutinin (PHA-P, Difco) was added at a dilution of 1·600, pokeweed mitogen (PWM, Gibco) at 1:500; concanavalin A (Con A, Pharmacia) was employed at a concentration of 20 μg/500 μl.

Standard mitogen stimulated cultures were harvested at 72 hours. For time sequence studies, cultures were harvested at 24, 48, 72 and 96 hours.

Microbial Antigens

Heat-killed (70° C, 30 min) *Staphylococcus aureus* and *Escherichia coli* were kindly prepared by Dr. *Klaus Jensen* and employed at concentrations of 5×10^5 and 5×10^4 per 500 μl. An extract of *Candida albicans* was kindly donated by Dr. *Niels Axelsen* (1) and employed at protein concentrations of 1000 and 100 μg per 500 μl. PPD without chinosol (Statens Seruminstitut) was used in concentrations of 5 and 0 5 μg per 500 μl.

Antigen-stimulated cultures were harvested at 120 hours.

Mixed Lymphocyte Culture

Mixed lymphocyte culture (MLC) was performed as previously described (7). In all cases, lymphocytes from unrelated donors, differing at two HL-A haplotypes, were used as stimulating cells.

Labelling and Harvest of Cultures

To all cultures, 0.05 μCi ^{14}C-thymidine per 500 μl was added 24 hours before termination. The cells were harvested on Whatman GF/C glassfiber filters and washed in distilled water. To the filters 10 ml of Instagel® were added, and they were counted in a Beckmann liquid scintillation counter. Individual counts were within ± 7 per cent of the mean in triplicate mitogen-stimulated cultures and within ± 10 per cent of the mean in antigen-stimulated cultures.

Values given are counts per minute (c.p m.) in stimulated cultures—c.p.m. in unstimulated cultures.

Serum immunoglobulin concentrations were determined by conventional techniques (9, 10).

RESULTS

The history of the thrombocytopenic family members studied strongly suggested a decreased resistance to infections; these were usually, but not always, of moderate severity and not life-threatening (Table 1), in most cases respiratory tract infections due to a variety of bacteria. Investigation of the immune system revealed lymphocyte concentrations in the lower normal range; IgM concentration was below normal in 5 and IgA concentration slightly above normal in 3 (Table 1). The concentrations of neutrophils and eosinophils in the blood were normal in all except one (Patient No. 95) who had an eosinophil concentration of 1150 millions/l. Thus, these investigations did not explain the increased propensity for infections.

The results of mitogen stimulation of lymphocytes are depicted in Fig. 1. All thrombocytopenic patients except one and all relatives showed results within the normal range. The single exception was Patient No. 94 who on two separate occasions showed low responses to PHA and PWM. Fig. 1 demonstrates that a subnormal response was seen at all time intervals tested; thus, there was no evidence of a delay in response.

Table 2 summarizes the results of lymphocyte stimulation with microbial antigens in the 6 patients so studied. All of these showed subnormal responses to one or more of the microbial antigenic systems shown in Fig. 2 ($p<.05$, rank comparison test with normal

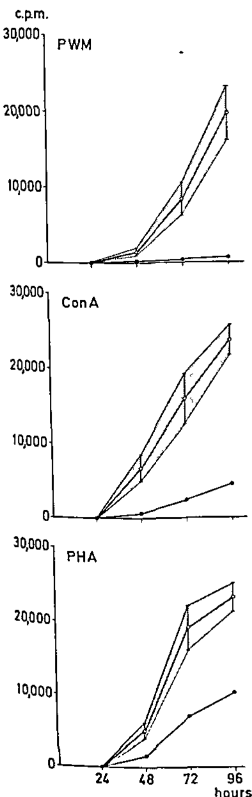


Fig. 1. Time course of lymphocyte transformation induced by mitogens (PWM = poke weed mitogen, Con A = concanavalin A, PHA = phytohaemagglutinin). Shaded area: Mean ± S.E.M. in normal subjects. ●—● Patient No. 94. All other persons included in this study showed results within the normal range.

---

cells); only with *Staphylococcus aureus*, the results obtained with the patients' lymphocytes corresponded to the transformation seen if normal lymphocytes were used. The response in MLC to allogeneic cells was also subnormal ($p < .05$) (Fig. 2).

Antigenic stimulation of the lymphocytes obtained from the patients' relatives showed that the responses to the extract of *Candida albicans* and to PPD were significantly lower in the relatives than in the normal controls (Table 2); with *Candida albicans*, approximately half of the relatives showed results as low as those of the thrombocytopenic patients (Fig. 2). The low responders were evenly distributed within the family, and it was not possible to correlate low response and presumed carrier state.

DISCUSSION

The disorder affecting the present family is clearly distinguished from the classical Wiskott-Aldrich syndrome in that almost all of the afflicted members in Wiskott-Aldrich families succumb during early childhood if not treated, and a high proportion of malignancies, in particular of the lymphoreticular system, is observed in those who survive into the second decade. Immune disability of comparable severity was not observed in the family examined in the present study, but several of the clinical features and laboratory findings suggesting the Wiskott-Aldrich syndrome were present in milder form in afflicted members.

The most important immunological abnormalities in the Wiskott-Aldrich syndrome are: Decreased antibody formation to a variety of infectious agents and in particular to polysaccharide antigens (2); increased cata-

TABLE 2. *Lymphocyte Transformation* in vitro

		CA	SA	EC	PPD	MLC	unstimulated
Patients	65*	1444	9562	717	1598	4127	251
	69	812	6291	1088	2050	4484	237
	78	804	n.d.	n.d.	383	466	299
	94	1760	4522	1585	626	4199	153
	102	451	4146	515	339	4146	267
	120	889	4166	2085	299	1158	291
Relatives	mean	2670	8768	2101	3489	6498	348
(90 per cent range)		(632–5612)	(3113–19636)	(1179–3600)	(1340–6086)	(1908–16380)	(154–532)
		$p<.001$	$p>.1$	$p>.05$	$p<.01$	$p>.5$	$p>.5$
Controls	mean	5184	5270	2827	5895	6205	410
(90 per cent range)		(2684–11104)	(1530–9062)	(1249–5404)	(1078–13795)	(2296–12334)	(109–611)

All values are counts per minute (c.p.m.). *Numbers as in Table 1.
PHA = phytohaemagglutinin, PWM = poke weed mitogen, CA = *Candida albicans* extract, SA = *Staphylococcus aureus*, EC = *Escherichia coli*, PPD = purified protein derivative, MLC = mixed lymphocyte culture. n.d. = not done.
The p values indicate the significance of the difference in responses between the relatives and the normal controls (Wilcoxon rank sum test).

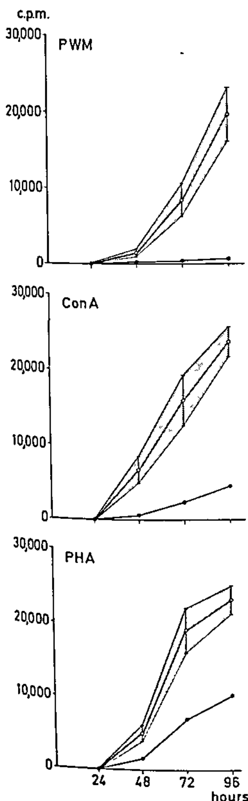


Fig. 1. Time course of lymphocyte transformation induced by mitogens (PWM = poke weed mitogen, Con A = concanavalin A, PHA = phytohaemagglutinin). Shaded area: Mean ± S.E.M. in normal subjects. ●—● Patient No. 94. All other persons included in this study showed results within the normal range.

cells); only with *Staphylococcus aureus*, the results obtained with the patients' lymphocytes corresponded to the transformation seen if normal lymphocytes were used. The response in MLC to allogeneic cells was also subnormal ($p < .05$) (Fig. 2).

Antigenic stimulation of the lymphocytes obtained from the patients' relatives showed that the responses to the extract of *Candida albicans* and to PPD were significantly lower in the relatives than in the normal controls (Table 2); with *Candida albicans*, approximately half of the relatives showed results as low as those of the thrombocytopenic patients (Fig. 2). The low responders were evenly distributed within the family, and it was not possible to correlate low response and presumed carrier state.

DISCUSSION

The disorder affecting the present family is clearly distinguished from the classical Wiskott-Aldrich syndrome in that almost all of the afflicted members in Wiskott-Aldrich families succumb during early childhood if not treated, and a high proportion of malignancies, in particular of the lymphoreticular system, is observed in those who survive into the second decade. Immune disability of comparable severity was not observed in the family examined in the present study, but several of the clinical features and laboratory findings suggesting the Wiskott-Aldrich syndrome were present in milder form in afflicted members.

The most important immunological abnormalities in the Wiskott-Aldrich syndrome are: Decreased antibody formation to a variety of infectious agents and in particular to polysaccharide antigens (2); increased cata-

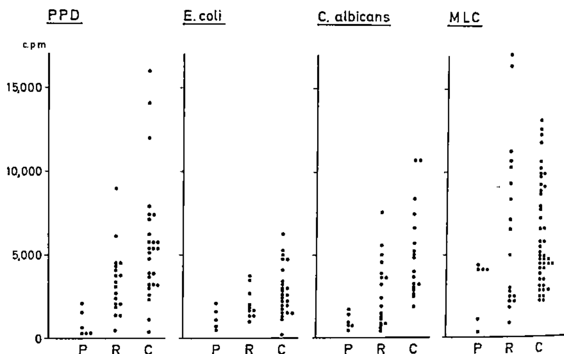


Fig. 2. Lymphocyte transformation induced by antigens and by allogeneic lymphocytes.
P: Patients. R: Relatives. C: Controls.

bolism of immunoglobulins with resultant decrease in IgM concentration, but due to an even more increased rate of synthesis of IgA generally elevated levels of this immunoglobulin (3). Lymphocytopenia may be present, and T-lymphocyte function is deficient (6); the lymphocyte transformation response to several soluble microbial antigens and to allogeneic lymphocytes is impaired (11), and lymphocyte production of migration inhibitory factor (MIF) is deficient (14, 12). A defect in the receptors for IgG on monocytes has been associated with clinical improvement after treatment with transfer factor, obtained in approximately half the patients (12).

In the thrombocytopenic patients included in this study, the lymphocyte and IgM concentrations were generally low, and the IgA concentrations tended to be high; on the basis of these routine examinations a clear separation of the patients from the normal was not possible. The lymphocyte transformation studies, however, illustrate some of the possibilities inherent in a more detailed investigation of the immune apparatus, aiming at a more exact localization and quantitiation of immunological deficiency. The most pronounced deficiency found was a severely reduced lymphocyte transformation in response to several microbial antigens; similarly, this is the earliest detectable lymphocyte defect during the evolution of malignant lymphoma (13). Two patients showed subnormal responses to allogeneic lymphocytes. Only one patient showed a reduced response to non-specific mitogens, but further discrimination may be possible by measuring the response to sub-optimal doses of PHA since this may be deficient in cases where optimal doses of PHA evoke a normal response (11). The pattern of *in vitro* lymphocyte responses encountered in our patients is similar to that observed in one patient with hereditary thrombocytopenia reported by *Weiden* & *Blaese* (15).

Of practical urgency is the problem of evaluating the risks involved in splenectomy on patients with thrombocytopenia (15). A rise in thrombocyte concentrations to normal

or near-normal levels was observed in all 5 members of the present family who underwent splenectomy. However, two developed severe infections and died one and seven years, respectively, following splenectomy (5), and patient No. 94 in the present study had a stormy postoperative course with life-threatening infection. It has been clearly established that the risk of fatal infection following splenectomy is dependent on the underlying condition, a high lethality being observed in *e.g.* Wiskott-Aldrich patients (8). A closer scrutiny of the thrombocytopenic patients presented here revealed evidence of immune deficiency, indicating the necessity for immunological investigation of patients with thrombocytopenia, in particular those with a family history suggesting a hereditary disorder.

This work was supported by grants from *Statens lægevidenskabelige forskningsråd, Den lægevidenskabelige forskningsfond for Storkøbenhavn, Færøerne og Grønland, Daell Fonden* og *F. L. Smidths Jubilæumsfond.*

REFERENCES

1. *Axelsen, N. H.:* Antigen-antibody crossed electrophoresis (Laurell) applied to the study of the antigenic structure of *Candida albicans.* Infect. Immun. *4:* 525–527, 1971.
2. *Blaese, R. M., Strober, W., Brown, R. S. & Waldmann, T. A.:* The Wiskott-Aldrich syndrome. A disorder with a possible defect in antigen processing or recognition. Lancet *1:* 1056–1061, 1968.
3. *Blaese, R. M., Strober, W., Levy, A. L. & Waldmann, T. A.:* Hypercatabolism of IgG, IgA, IgM, and albumin in the Wiskott-Aldrich syndrome. J. Clin. Invest. *50:* 2331–2338, 1971.
4. *Canales, L. & Mauer, A. M.:* Sex-linked hereditary thrombocytopenia as a variant of Wiskott-Aldrich syndrome. New Engl. J. Med. *277:* 899–901, 1967.
5. *Cohn, J., Andersen, V., Thomsen, M., Staub Nielsen, L., Hauge, M., Henningsen, K. & Iversen, T.:* Sex-linked hereditary thrombocytopenia with immunologic defects. Human Heredity (in press).
6. *Cooper, M. D., Chase, H. P., Lowman, J. T., Krivit, W. & Good, R. A.:* Wiskott-Aldrich syndrome. An immunologic deficiency disease involving the afferent limb of immunity. Amer. J. Med. *44:* 499–513, 1968.
7. *Dupont, B., Andersen, V., Hansen, G. S. & Svejgaard, A.:* Abnormal mixed lymphocyte reactions in the family of a patient with severe combined immunodeficiency. Transplantation *14:* 557–563, 1972.
8. *Eraklis, A. J., Kevy, S. V., Diamond, L. K. & Gross, R. E.:* Hazard of overwhelming infection after splenectomy in childhood. New Engl. J. Med. *276:* 1225–1229, 1967.
9. *Johansson, S. G. O., Bennich, H. & Wide, L.:* A new class of immunoglobulin in human serum Immunology *14:* 265–272, 1968.
10. *Laurell, C.-B.:* Quantitative estimation of protein by electrophoresis in agarose gel containing antibodies. Anal. Biochem. *15:* 45–52, 1966.
11. *Oppenheim, J. J., Blaese, R. M. & Waldmann, T. A.:* Defective lymphocyte transformation and delayed hypersensitivity in Wiskott-Aldrich syndrome. J. Immunol. *104:* 835–844, 1970.
12. *Spitler, L. E., Levin, A. S., Stites, D. P., Fudenberg, H. H., Pirofsky, B., August, C. S., Stiehm, E. R., Hitzig, W. H. & Gatti, R. A.:* The Wiskott-Aldrich syndrome. Results of transfer factor therapy. J. Clin. Invest. *51:* 3216–3224, 1972.
13. *Thomsen, M.:* Unpublished studies.
14. *Thor, D.,* quoted in 11.
15. *Weiden, P. L. & Blaese, R. M.:* Hereditary thrombocytopenia: Relation to Wiskott-Aldrich syndrome with special reference to splenectomy. J. Pediatr. *80:* 226–234, 1972.

Acta path. microbiol. scand. Sect. C, *83:* 184–188, 1975

A REACTION BETWEEN SOME STREPTOCOCCI AND IgA MYELOMA PROTEINS

POUL CHRISTENSEN and VIVI-ANNE OXELIUS

The Department of Medical Microbiology, University of Lund, Sölvegatan 23, S-223 62 Lund, Sweden

Christensen, P. & Oxelius, V.-A. A reaction between some streptococci and IgA myeloma proteins. Acta path. microbiol. scand. Sect. C, *83:* 184–188, 1975.

A reaction is described between some streptococci and IgA myeloma proteins; most streptococci showed low affinity for IgA myeloma proteins, with the exception of group A, type M 4 and two freshly isolated group A streptococci; they took up 40 to 50 per cent of 1 μg ^{125}I labelled IgA myeloma protein added. Addition of similar amounts of unlabelled IgA and IgG myeloma proteins showed no cross inhibition between uptake of IgG and IgA. Heat- and trypsin treatment of the streptococci did not reveal any differences in IgA and IgG reactivity. The uptake of one ^{125}I labelled IgA myeloma protein was inhibited uniformely by four randomly selected unlabelled IgA myeloma proteins. Thus, the uptake of ^{125}I labelled IgA myeloma protein seems to be independent from the specificity of the antibody combining sites.

Key words: IgA myeloma proteins; streptococci.

P. Christensen, The Department of Medical Microbiology, Solvegatan 23, S-223 62 Lund, Sweden.

Received 17.x.74 Accepted 6.xii.74

The reaction of protein A from *Staphylococcus aureus* with the Fc portion of IgG was reported in 1966 by *Forsgren & Sjöquist;* recently, a binding of 10 of 32 monoclonal human IgM proteins to the *S. aureus* has been described (*Harboe & Fölling* 1974). Furthermore, an interaction has been demonstrated between IgG and some streptococci, irrespective of the antibody combining sites (*Kronvall* 1973, *Christensen & Kronvall* 1974, *Christensen & Oxelius* 1974) and between IgG and pneumococci (*Stephens et al.* 1974).

This paper concerns the capacity of some streptococci to react with IgA myeloma proteins; the uptake of ^{125}I labelled purified IgA myeloma protein by the streptococci was measured in the way described for quantitation of the uptake of IgG by streptococci (*Christensen & Oxelius* 1974).

MATERIALS AND METHODS

Streptococcal Strains

The following group A streptococci, kindly supplied by the Central Public Health Laboratories, London, were studied: M 1 (8198), M 2 (8322), M 3 (100064), M 4 (SS 241), M 5 (100065), M 6 (8302), M 8 (8324), M 9 (100067), M 11 (100068), M 12 (100085), M 15 (100070), M 17 (8304), M 49 (10076), M 56 (100191), and M 57 (100190).

The group B, C, D and G strains and the other group A streptococci used in the experiments were isolated from routine bacteriological specimens;

they were grouped as described previously (*Christensen et al.* 1973). These strains were tested for uptake of IgA myeloma protein directly after isolation.

The streptococci were cultured in Todd Hewitt broth. A standard suspension of 2.5×10^{10} streptococci/ml PBS (phosphate buffered saline, 0.12 M NaCl, 0.03 M phosphate, pH 7.2) was prepared as described previously (*Christensen* & *Oxelius* 1974).

Human Immunoglobulin Preparations

IgG myeloma protein was purified from one human serum in the way described earlier (*Christensen* & *Oxelius* 1974). IgA myeloma proteins (termed No. 128, 129, 142 and 151) were isolated from four human sera by ammonium sulphate precipitation, preparative electrophoresis in agarose (*Laurell* 1965) and gel filtration (*Flodin* & *Killander* 1962).

Solutions of the purified myeloma proteins were prepared in PBS. The protein was measured with a modification of *Folin*'s method (*Lowry et al.* 1951).

Determination of the Uptake of IgG and IgA by Streptococci

One of the purified IgA myeloma proteins, No. 128, and the IgG myeloma protein were labelled with ^{125}I as described by *McConahey* & *Dixon* (1966). The uptake of IgG and IgA by 0.2 ml standard suspension of streptococci was measured as described earlier (*Christensen* & *Oxelius* 1974).

Trypsin and heat treatment of streptococci was performed as described previously (*Christensen* & *Oxelius* 1974).

RESULTS

The Capacity of Some Selected Streptococci to Take up ^{125}I Labelled Purified IgA and IgG Myeloma Proteins

The uptake of ^{125}I labelled IgA and IgG myeloma proteins (in per cent of 1 μg added) by some group A streptococci of different M types is given in Fig. 1. Type M 1 took up 50 per cent IgG, but only 10 per cent IgA; on the other hand, M 4 took up 44 per cent IgA and 17 per cent IgG. With these exceptions, the uptake of IgG as well as of IgA was below 30 per cent.

The uptake of ^{125}I labelled IgA myeloma protein by 13 group A strains, 14 B, 3 C, 5 D and 3 G, freshly isolated from routine bacteriological specimens is shown in Fig. 2. Two of the group A strains (termed 170 A and 313 A) took up 40 to 50 per cent IgA.

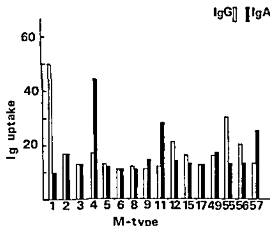


Fig. 1. The uptake of ^{125}I labelled IgA and IgG myeloma proteins by different M- types of group A streptococci. The uptake is given in per cent of 1 μg ^{125}I labelled Ig added.

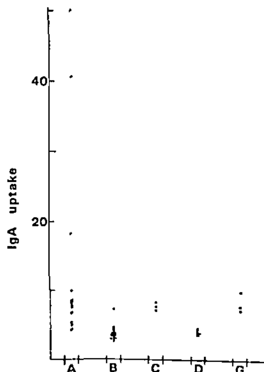


Fig 2. The uptake of ^{125}I labelled IgA myeloma protein by some streptococci freshly isolated from routine bacteriological specimens. The uptake is given in per cent of 1 μg ^{125}I labelled Ig added.

Relation of the Uptake of IgA by Streptococci to the Uptake of IgG

The uptake of ^{125}I labelled myeloma protein by some streptococci was measured in inhibition experiments with unlabelled myeloma proteins; 1, 5 or 10 μg unlabelled myeloma protein was mixed with 1.0 μg labelled myeloma protein (total volume 250 μl), after which 0.2 ml streptococcal standard suspension was added.

In contrast to the effect of unlabelled IgG, addition of IgA had only a slight inhibitory effect on the uptake of ^{125}I labelled IgG by streptococcus group B, strain 123 B (Fig. 3). On the other hand, the uptake of ^{125}I labelled IgA by streptococcus group A, type M 4 was substantially inhibited by unlabelled IgA, but not by IgG (Fig. 4).

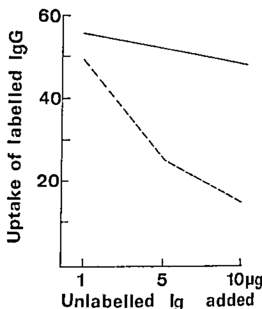


Fig. 3. Inhibition of the uptake of ^{125}I labelled IgG myeloma protein by streptococcus group B, strain 123 B, by addition of unlabelled IgA and IgG myeloma proteins. Solid line: IgA; dotted line IgG The uptake is given in per cent of 1 μg ^{125}I labelled Ig added.

Inhibition of the Uptake of ^{125}I Labelled IgA Myeloma Protein (No. 128) by Streptococci Group A by Addition of Other Purified IgA Myeloma Proteins

Unlabelled purified IgA myeloma proteins (No. 128, 129, 142 or 151), 1, 5 or 10 μg (total volume 250 μl) and ^{125}I labelled purified IgA myeloma protein (No. 128) (1 μg) were mixed after which 0.2 ml standard suspension of streptococcus group A, type M 4, group A, strain 170 A or group A, strain 313 A was added. The unlabelled myeloma proteins, No. 129, 142 and 151 inhibited the uptake of ^{125}I labelled myeloma protein No. 128 on M 4, 170 A and 313 A streptococci as much as did the unlabelled myeloma protein No. 128.

Influence of Heating and Trypsin Treatment of Streptococci on the Uptake of IgA and IgG

Type M 1, type M 4, one group B strain and one group C strain were heated for 30 min at 56° C or for 15 min at 100° C. The mean values found for the uptake of IgG or IgA by the four strains are given in Table 1. The uptake of IgG and IgA was somewhat less after heating at 100° C for 15 min; the IgA uptake was slightly suppressed by heating for 30 min at 56° C.

TABLE 1. *The Effect of Heating the Streptococci on the Uptake of ^{125}I Labelled IgA and IgG Myeloma Protein*

Treatment	Mean uptake of ^{125}I labelled IgA*	Mean uptake of ^{125}I labelled IgG*
Stored at 4° C	22.3	30.6
Heated at 56° C for 30 min	17.0	31.1
Heated at 100° C for 15 min	16.2	27.3

* The uptake by 0.2 ml standard suspension of the streptococci is given in per cent of 1 μg added. Mean values for four strains, group A, type M 1, type M 4, one group B strain and one group C strain, are shown.

The mean uptake of IgG and IgA after 18 hours' treatment with trypsin was about half that noted without trypsin treatment.

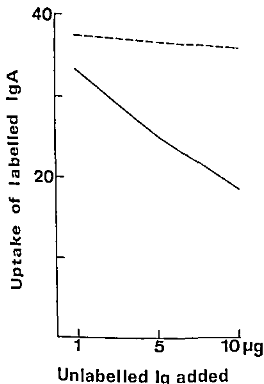


Fig. 4. Inhibition of the uptake of ^{125}I labelled IgA myeloma protein by streptococcus group A, type M 4, by addition of unlabelled IgA and IgG myeloma proteins. Solid line: IgA; dotted line IgG. The uptake is given in per cent of 1 µg ^{125}I labelled Ig added.

DISCUSSION

A reaction between some streptococci and IgA was demonstrated in the present investigation. The results obtained by the use of purified myeloma proteins indicated that the probability of participation of the antibody combining sites in the reaction was minimal; the myeloma protein from each individual is synthesized by a clone of identical cells derived from one original cell. Thus, the reaction between IgA myeloma protein and the streptococci seems to be independent from the specificity of the antibody combining sites. The data given in Fig. 3 and Fig. 4 indicated no competition between the uptake of IgG and IgA, respectively, on the streptococci. It would be valuable to define the structures on the streptococci and on the IgA and IgG, respectively responsible for the absorption of these two immunoglobulins.

The biological significance of this streptococcal reactivity with IgA is with our present knowledge obscure. A relation of this reactivity to the function of S- IgA, suggested by *Gibbons* (*Gibbons* & *van Houte* 1971, *Ellen* & *Gibbons* 1972, *Williams* & *Gibbons* 1972), to sterically hinder the attachment of the streptococci to epithelial cells might be of interest and demands further investigations.

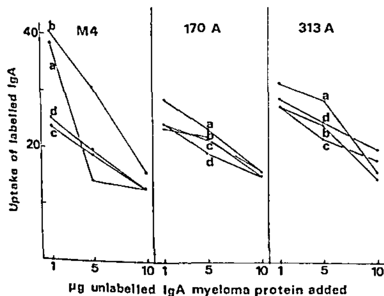


Fig. 5. Inhibition of the uptake of ^{125}I labelled IgA myeloma protein by 3 different group A streptococci, by addition of 4 different purified IgA myeloma proteins. a: IgA myeloma protein No. 128; b: No. 129; c: No. 142; and d: No. 151. The uptake is given in per cent of 1 µg ^{125}I labelled Ig added.

REFERENCES

Christensen, P., Kahlmeter, G., Jonsson, S. & *Kronvall, G.:* New method for the serological grouping of streptococci with specific antibodies adsorbed to protein A-containing staphylococci. Infec. Immun. *7:* 881–885, 1973.

Christensen, P. & *Kronvall, G.:* Capacity of group A, B, C, D and G streptococci to agglutinate sensitized sheep red cells. Acta path. microbiol. scand. Sect. B, *82:* 19–24, 1974.

Christensen, P. & *Oxelius, V.-A.:* Quantitation of the uptake of human IgG by some streptococci groups A, B, C and G. Acta path. microbiol. scand. Sect. B, *82: 475–483, 1974.*

Ellen, R. P. & *Gibbons, J.:* M protein -associated adherence of ***Streptococcus pyogenes*** to epithelial surfaces: prerequisite for virulence. Infec. Immun. *5:* 826–830, 1972.

Flodin, P. & *Killander, J.:* Fractionation of human serum proteins by gel filtration. Biochem. biophys. Acta (Amst.) *63:* 403–410, 1962.

Forsgren, A. & *Sjöquist, J.:* Protein A from *S. aureus.* I. Pseudo-immune reaction with human γ-globulin. J. Immunol. *97:* 822–827, 1966.

Gibbons, R. J. & *van Houte, J.:* Selective bacterial adherence to oral epithelial surfaces and its role as an ecological determinant. Infec. Immun. *3:* 567–573, 1971.

Harboe, M. & *Fölling, I.:* Subclasses of IgM based on differences in reactivity with ***Staphylococcus aureus*** (Abstract). V annual meeting of the scandinavian society for immunology. Copenhagen, May 29–31, 1974, p. 47.

Kronvall, G.: A surface component in group A, C and G streptococci with non-immune reactivity for immunoglobulin G. J. Immunol. *111:* 1401–1406, 1973.

Laurell, C.-B.: Antigen-antibody crossed electrophoresis. Anal. Biochem. *10:* 358–361, 1965.

Lowry, O. H., Rosebrough, N. J., Farr, L. A. & *Randall, R. J.:* Protein measurement with the Folin phenol reagent. J. Biol. Chem. *73:* 637–645, *1951.*

McConahey, P. J. & *Dixon, F. J.:* A method of trace iodination of proteins for immunologic studies. Int. Arch. Allergy *29:* 185–189, 1966.

Stephens, C. G., Reed, W. P., Kronvall, G. & *Williams, R. C., Jr.:* Reactions between certain strains of pneumococci and Fc of IgG. J. Immunol. *112:* 1955–1960, 1974.

Williams, R. C. & *Gibbons, R. J.:* Inhibition of bacterial adherence by secretory immunoglobulin A: a mechanism of antigen disposal. Science *177:* 697–699, 1972.

Acta path. microbiol. scand. Sect. C, 83: 189–194, 1975

IgG ANTIBODIES TO IgA IN TWO PATIENTS WITH HYPOGAMMAGLOBULINAEMIA TREATED WITH COMMERCIAL GAMMAGLOBULIN

CARL KAMME*, ERIK DAHLQUIST**, SVANTE JONSSON* and FOLKE LINDSTRÖM***

Departments of Medical Microbiology* and Infectious Diseases**, University Hospital, Lund, and Department of Medicine***, University Hospital, Linkoping, Sweden

Kamme, C., Dahlquist, E., Jonsson, S. & Lindström, F. IgG antibodies to IgA in two patients with hypogammaglobulinaemia treated with commercial gammaglobulin. Acta path. microbiol. scand. Sect. C, 83: 189–194, 1975.

Antibodies to IgA were detected in two males with hypogammaglobulinaemia. The antibodies were demonstrated to belong to the IgG class. In both patients low serum levels of IgG and IgM but no IgA were found. On two occasions one of the patients reacted with severe side reactions within a few seconds after an intramuscularly given injection of gammaglobulin. The other patient had received monthly intramuscular injections of gammaglobulin over a period of 2 years when the anti-IgA antibodies first appeared and has since then been regularly treated for 1½ years without any signs of adverse reactions. In five additional patients with hypogammaglobulinaemia and side reactions after i.m. injections of gammaglobulin no antibodies to IgA, IgG or Gm-determinants were detected.

Key words: Hypogammaglobulinaemia; gammaglobulin treatment; IgG antibodies to IgA.

C. Kamme, Dept. of Medical Microbiology, Solvegatan 23, 22362 Lund, Sweden.

Received 23.x.74 Accepted 1.xii 74

Intramuscular administration of gammaglobulin to patients with hypogammaglobulinaemia may sometimes give rise to systemic side effect (6, 11, 22). According to the report of the Medical Research Council of the United Kingdom untoward reactions appeared after 85 out of 40 000 i.m. injections, given over 11 years and affected 32 (18 per cent) of 175 patients (22).

The process of manufacturing commercial preparations of human gammaglobulin involves some degree of denaturation in the form of aggregates (6). Aggregated IgG possesses biologic activities that are absent in native IgG. These activities include complement fixation and the ability to provoke skin reactions in guinea pigs and in man (8, 9, 18).

On i.v. administration, the side effect can be correlated to the complement-binding activity of the preparations used, and a consumption of complement can be detected. Patients with primary hypogammaglobulinaemia seem to be more sensitive to the toxic effect of gammaglobulin aggregates than are healthy individuals and patients with secondary hypogammaglobulinaemia (2, 3).

The adverse reactions following i.m. administration of human gammaglobulin to patients with hypogammaglobulinaemia may be caused by either aggregated IgG or an antigen-antibody interaction. With the exception of one patient with antibodies against aggregated IgG (7), no evidence has been presented suggesting that antigammaglobulin antibodies are involved in the side reactions. A consumption of complement has not been demonstrated in association with reactions following i.m. injections (22).

In recent years attention has been directed to class specific anti-IgA antibodies which may appear in subjects with selective IgA deficiency and cause severe side reactions on administration of blood or plasma (4, 13, 16, 19). It has been pointed out that the amount of IgA in a few ml of red cell concentrate is sufficient to produce a severe reaction in those individuals (16). The present report presents evidence that a production of antibodies to IgA may occur in patients with hypogammaglobulinaemia as an immune response to the IgA in the administered gammaglobulin.

MATERIAL AND METHODS

IgG was prepared from pooled human sera by starch block electrophoresis (5). IgG was aggregated with bisdiazotized benzidin by the method of *Ishizaka & Ishizaka* (9) or by heating of a solution of 10 mg/ml at +63° C for 20 minutes (7).

IgA was prepared from pooled human sera by the method of *Tomasi & Bienenstork* (23).

Cohn fraction II prepared from pooled human sera was kindly supplied by AB Kabi, Stockholm, Sweden. About 3 per cent of the total immunoglobulin content consisted of IgA as determined by electroimmuno-assay.

Commercial rabbit antihuman IgA, anti-IgG and anti-IgM antisera were used (Hyland laboratories and Behringwerke).

Gel diffusion, immunoelectrophoresis and the Waaler-Rose test were performed according to conventional methods.

Quantitative determination of immunoglobulins was done according to *Oudin*, with electroimmunoassay or with radial immunodiffusion according to *Mancini* (14, 17, 20).

Absorption of IgG in patients' sera by protein A-containing *Staphylococcus aureus* was performed with bacteria of the Cowan I strain, treated with formaldehyde and heat according to *Jonsson & Kronvall* (10). Two ml of a 10 per cent suspension were centrifuged, the supernatant discarded, and the pellet resuspended in 1 ml of the patients' serum, diluted 1/20 in phosphate-buffered saline (PBS), pH 6.4. The mixture was kept at room temperature for 15 minutes, and the bacteria were then spun down at 3 000 × *g* for 30 minutes.

The preparation of insolubilized anti-IgA, anti-IgG and anti-IgM antisera for use as reversed immunosorbents was done according to *Avrameas & Ternynck* (1). One ml of patients' serum, diluted 1/20 in PBS, pH 6.4, was added to the centrifuged pellet of immunosorbent prepared from 1 ml rabbit antiserum or normal rabbit serum. The mixture was incubated at room temperature overnight with slow rotation. The tubes were then centrifuged and the supernatant subjected to one further centrifugation before assay.

Gel filtration was performed on a column packed with Sephadex G-200 (Pharmacia) in saline buffered with 0.015 M triethanolamine, pH 7.4.

The agglutination test of human erythrocytes coated with incomplete anti Rh-antibodies included the anti-Rh Ri (24).

The indirect haemagglutination test (IHA) for assay of anti-IgA and anti-IgG antibodies was performed with formalinized (25) tanned sheep erythrocytes. The formalinized cells were treated with tannic acid 1:40 000 at +56° C in PBS, pH 7.2 for 30 minutes. Adsorption of the immunoglobulins to the cells was performed at room temperature for 20 minutes in PBS, pH 6.4 To 4 ml of the gammaglobulin solution, tanned erythrocytes were added to a final concentration of 5 × 10^9 cells/ml. The concentration of IgG and IgA varied from 0.5–2.0 mg/ml. 0.02–0.08 mg/ml of aggregated IgG usually gave satisfactory coating. Of the Cohn II fraction 2 mg/ml was used.

After coating, the cells were spun down, resuspended to a concentration of 3 per cent in PBS, pH 6.4, and then used in the test. The coated cells could be stored at +4° C for 7 days without loss of quality.

The anti-IgA and anti-IgG antisera were made specific by absorption.

The IHA test and the haemagglutination inhibition test (IHAI) were carried out as previously described (12).

Patients

The patients consisted of seven cases with hypogammaglobulinaemia; six men aged 17–53 years and one woman, 40 years old. Six of them had adverse reactions to i.m. injections of commercial gammaglobulin and came to our knowledge for that reason.

Anti-IgA antibodies were found in two patients, who are described under case reports

RESULTS

Case Reports

Case 1. G.B. A man born in 1936. Mainly free from infections until about 20 years of age, when he began to have recurrent respiratory tract infections. Over a period of 12 years (1956–1968) he had pneumonia on about 20 occasions. Hypogammaglobulinaemia was diagnosed 1968. Immunoglobulins in serum: IgG 110 mg per cent, IgM 8.8 mg per cent, IgA < 0.1 mg per cent. The serum levels of the immunoglobulins have not changed significantly since 1968.

Intramuscular administration of 10 ml 16.5 per cent "Gammaglobulin Kabi" (AB Kabi, Stockholm, Sweden) was started in September 1968 with one injection every fortnight. He received this treatment for one year without signs of untoward reactions. In september 1969, a few seconds after the injection was given, he reacted with dyspnoe, tachycardia, feeling of oppression in the chest and anxiety. No fall in blood pressure was noted. The symptoms lasted for about 1 hour. The following 3 injections, given with intervals of 1 month, were not associated with any side reactions.

In January 1970, a few seconds after receiving 10 ml gammaglobulin, he reacted with the same symptoms as in September 1969, but they were now more intense and lasted for about 6 hours. Because of this severe reaction he has refused further gammaglobulin treatment. The first available serum from the patient was taken in January 1970 a few minutes prior to the injection, that was followed by the reaction. The specificity of the antibodies found is demonstrated in Table 1. Unfortunately no serum sample could be collected from the patient until 4 months later. Altogether 7 serum samples, collected over a period of 14 months after he refused further treatment, were examined. The antibody titre remained unchanged over this period.

Case 2. O.H. A man born in 1927. Mainly free from infections until about 25 years of age, when he began to have recurrent respiratory tract infections. 1954–1963 he had pneumonia on 6 and maxillary sinusitis on 3 occasions.

Hypogammaglobulinaemia was diagnosed 1963: IgG in serum 220 mg per cent. Immunoglobulins in serum 1970: IgG 250 mg per cent, IgM 9 mg per cent, IgA < 0.1 mg per cent. The serum levels of the immunoglobulins have not changed significantly since 1970. From 1963 to 1966 he was treated with monthly injections of 24 ml 12 per cent "Gammaglobulin Kabi". Over this period, when he received about 35 injections without any complications, he was free from infections. The treatment was stopped in spring 1966. In summer 1969 he had a pneumonia and the gammaglobulin therapy started again. He has since received monthly injections of 20 ml 16.5 per cent "Gammaglobulin Kabi" without any signs of adverse reactions and he has largely been free from infections.

The first serum available from the patient was collected in August 1970 and serum samples have since been regularly collected each month.

TABLE 1. *Agglutination with Test Antisera and Patients' Sera of Sheep Erythrocytes Coated with IgA, IgG and Cohn Fraction II*

Coats	IgA	IgG	Aggregated IgG	Cohn fraction II
Anti-IgA*	3200 6400	< 40	< 40	3200
Anti-IgG*	< 40	1600–3200	3200–6400	3200
Case 1 (G.B.)	640–1280	< 20	< 20	320–640
Case 2 (O.H.)	160–320	< 20	< 20	160–320
Cases 3–7	< 20	< 20	< 20	< 20

Titres expressed as reciprocals of serum dilution.
* Rabbit antisera.

Over the period August 1970 – July 1972 no antibodies (titre < 1/20) were found in altogether 24 serum samples examined. The antibodies appeared in the serum collected one month later, and after that occasion the titre has remained unchanged over a period of 18 months. The specificity of the antibodies is demonstrated in Table 1.

Laboratory Investigation

The specificity of the IHA-test system and of the antibodies found in the patients is given in Table 1. The anti-IgA antibodies found in cases 1 and 2 could not be detected in gel diffusion tests or in immunoelectrophoresis with varying concentration of IgA as antigen.

Sera from all patients were negative in the Waaler-Rose test and in tests with human erythrocytes coated with incomplete anti Rh-antibodies.

IgA and IgG preparations purified in the laboratory varied somewhat in capacity to coat the tanned sheep erythrocytes. However, using the Cohn fraction II as antigen the titres in IHA could be reproduced within one dilution step. For this reason cells coated with Cohn fraction II were included in the tests. The specificity of the anti-IgA antibodies could be determined in the IHAI test by use of these cells as antigen and human sera with varying levels of IgA as inhibitors (Table 2).

TABLE 2. *Inhibition of Agglutination of Sheep Erythrocytes Coated with Cohn Fraction II in Tests with 5–10 Agglutinating Units of Human Anti-IgA Serum (Case 1)*

Inhibitors	Concentration of IgA* in undiluted inhibitor (µg/ml)	Concentration of IgA in highest dilution giving inhibition (µg/ml)
Human serum 1	820	0.05–0.1
Human serum 2	< 1	No inhibition
Human serum 3	4800	0.04–0 08
Human serum 4	1700	0.05–0.1
Human serum 5	60	0.07–0.14

* Determined by electro-immuno assay.

Fifteen additional human sera were used as inhibitors in the IHAI test. All sera inhibited 5–10 agglutinating units of the two human anti-IgA sera. This suggested that the anti-IgA antibodies were class-specific. The capacity to inhibit the reactions corresponded well to the IgA content of the fifteen sera.

Following gel filtration of the two patients' sera on Sephadex G-200 and concentration of the fractions the antibody activity when tested in IHA was recovered in the IgG fraction.

In aliquots of the two patients' sera which were absorbed with the Cowan I strain the titres in IHA were reduced to 1/20 or less. The absorption reduced the IgG levels with about 95 per cent but did not influence the IgM levels.

Further evidence that the anti-IgA antibodies were of the IgG class was obtained when using insolubilized rabbit anti-Ig antisera. Following absorption of the patients sera with anti-IgG, the IHA titre fell to less than 1/20 while no significant titre change was noted following absorption with anti-IgA, anti-IgM or normal rabbit serum (Table 3).

TABLE 3. *The IHA Titres Following Adsorption of the Two Patients' Anti-IgA Sera with Insolubilized Rabbit Antisera Against IgA, IgG and IgM*

	Patient No. 1	Patient No. 2
Before adsorption	640	320
Following adsorption with insolubilized		
anti-IgA	320	320
anti-IgG	< 20	< 20
anti-IgM	320	160
Normal rabbit serum	640	320

Titres expressed as reciprocals of serum dilution.

The presence of anti-IgA antibodies in cases 1 and 2 initiated an examination of the IgA content of the gammaglobulin preparations produced by different manufacturers. In all the 7 brands of human gammaglobulin for i.m. use, that are commercially available in Sweden, and also in the preparation for i.v.

REFERENCES

1. *Avrameas, S.* & *Ternynck, T.:* Biologically active water-insoluble protein polymers. J. Biol. Chem. *7:* 1651–1659, 1966.
2. *Barandun, S., Kaiser, M.* & *Bostal, W.:* Zur Frage der intravinösen gamma-globulin Applikation. Helv. med. Acta *28:* 551–555, 1961.
3. *Barandun, S., Kistler, P., Jeunet, F.* & *Isliker, H.:* Intravenous administration of human gamma-globulin. Vox Sang. (Basel) *7:* 157–174, 1962.
4. *Bjerrum, O. J.* & *Jersild, C.:* Class specific anti-IgA associated with severe anaphylactic transfusion reactions in a patient with pernicious anaemia. Vox Sang. *21:* 411–424, 1971.
5. *Bloemendel, H.:* Zone electrophoresis in blocks and columns. Elsevier Publishing Company, Amsterdam 1963, p. 42.
6. *Ellis, E. F.* & *Henney, C. S.:* Adverse reactions following administration of human gamma-globulin. J. Allerg. *43:* 45–54, 1969.
7. *Henney, C. S.* & *Ellis, E. F.:* Antibody production to aggregated human gamma G globulin in acquired hypogammaglobulinaemia. New Eng. J. Med. *278:* 1144–1146, 1968.
8. *Ishizaka, T.* & *Ishizaka, K.:* Biological activity of aggregated gamma-globulin. I. Skin reactive and complement fixing properties of heat denaturated gamma-globulin. Proc. Soc. exp. Biol. (N.Y.) *101:* 845–850, 1959.
9. *Ishizaka, K.* & *Ishizaka, T.:* Biological activity of aggregated gamma-globulin. II. A study of various methods for aggregation and species differences. J. Immunol. *85:* 163–171, 1960.
10. *Jonsson, S.* & *Kronvall, G.:* The use of protein A containing *Staphylococcus aureus* as a *solid phase anti-IgG reagent in radioimmuno*-assays as exemplified in the quantitation of α-fetoprotein in normal adult serum. Eur. J. Immunol. *4:* 29–33, 1974.
11. *Kamme, C., Coster, C., Hagelquist, E., Kalen, N., Lindholm, H.* & *Grubb, R.:* Side effects of treatment of hypogammaglobulinaemia with gamma-globulin. Acta med. scand. *179:* 679–690, 1966.
12. *Kamme, C.:* Serological grouping of staphylococcal phages by indirect haemagglutination. Acta path. microbiol. scand. Sect. B, *80:* 923–930, 1972.
13. *Nadorp, J. H. S., Voss, M., Buys, W. C., van Munster, P. J. J., van Tongaren, J. H. M., Aalberce, R. C.* & *van Loghem, E.:* The significance of the presence of anti-IgA antibodies in individuals with an IgA deficiency. Eur. J. clin. Invest. *3:* 317–323, 1973.
14. *Laurell, C. B.:* Electro-immuno assay. Scand. J. clin. Lab. Invest. *29:* 21–37, 1972. (Suppl. 124).
15. *Leikola, J., Fudenberg, H. H., Vyas, G. N.* & *Perkins, H. A.:* Isoantibodies to human IgM. Serological and immunochemical investigations. J. Immunol. *106:* 1147–1153, 1971.
16. *Leikola, J., Koistinen, J., Lehtinen, M.* & *Virolainen, M.:* IgA-induced anaphylactic transfusion reactions. A report of four cases. Blood *42:* 111–119, 1973.
17. *Mancini, G., Carbonara, A. D.* & *Hermans, J. F.: Immunochemical quantitation of anti*gens by single radial immunodiffusion. Int. J. Immunochem. *2:* 235–254, 1965.
18. *Marcus, D.:* A study of the mechanism of the anticomplementary activity of gamma-globulin. J. Immunol. *84:* 273–284, 1960.
19. *Miller, W. V., Holland, P. V, Sugarbaker, E, Strober, W.* & *Waldman, T. A.:* Anaphylactic reactions to IgA. A difficult transfusion problem. Amer. J. clin. path. *54:* 618–621, 1970.
20. *Oudin, J.:* Specific precipitation in gels and *its application to immunochemical* analysis. Meth. Med. Res. *5:* 335–359, 1952.
21. *Pretty, H. M., Fudenberg, H. H., Perkins, H. A.* & *Gerbode, F.:* Anti-γ globulin antibodies after open heart surgery. Blood *32:* 205–216, 1968.
22. *Soothill, J. F.:* Reactions to immunoglobulin. In: Hypogammaglobulinaemia in the United Kingdom. Medical Research Council. Special report series 310, p. 106. Her Majesty's stationery office, London 1971.
23. *Tomasi, T. B.* & *Bienenstock, J.:* Secretory immunoglobulins. Advances in immunology *9:* 1–96, 1968.
24. *Waller, M. V.* & *Vaughan, J. H.:* Use of anti-Rh sera for demonstrating agglutination activating factor in rheumatoid arthritis. Proc. Soc. Exp. Biol. (N.Y.) *92:* 198–204, 1956.
25. *Weinbach, R.:* Die Verwendbarkeit formolbehandelter Erythrocyten als Antigenträger in der indirekten Haemagglutination. Schweiz. Z. Path. *21:* 1043–1052, 1958.

Acta path. microbiol. scand. Sect. C, *83:* 195–202, 1975

INFLUENCE OF ENDOTOXIN AND COMPLEX BACTERIAL ANTIGENS IN THE PRESENCE OF SERUM FACTORS UPON THE FUNCTION OF HUMAN NEUTROPHILS *IN VITRO*

CHRISTIAN KOCH

The University Clinic for Infectious Diseases, and Statens Seruminstitut,
Department of Clinical Microbiology, Blegdamshospitalet, Copenhagen, Denmark

Koch, Chr. Influence of endotoxin and complex bacterial antigens in the presence of serum factors upon the function of human neutrophils *in vitro*. Acta path. microbiol. scand. Sect. C, 83: 195–202, 1975.

Endotoxin, added to normal human neutrophils in the presence of serum, simultaneously with the addition of *Staphylococcus aureus*, caused stimulation of ingestion as well as intracellular killing of the bacteria in doses ranging from 0.1 to 1000 µg endotoxin per ml. Stimulation of ingestion was also induced by simultaneous addition of complex microbial antigens, heat-killed bacteria, and precipitating as well as soluble human serum albumin (HSA)/anti-HSA complexes. On the other hand, inhibition of intracellular killing could be induced when the leucocytes were pre-incubated with endotoxin or precipitating HSA/anti-HSA complexes. High doses of complex microbial antigens, particularly in the presence of immune serum containing multiple specific precipitating antibodies, resulted in inhibition of intracellular killing after pre-incubation as well as by simultaneous addition to the neutrophils with the bacteria. It is suggested that the functional changes, induced by endotoxin and by microbial antigens, are secondary to ingestion of immune complexes. These changes are, however, not as pronounced as those previously demonstrated in circulating neutrophils from patients with severe bacterial infection. Direct interaction of endotoxin or microbial antigens with the neutrophils *in vivo*, is not solely responsible for the functional changes seen in patients with severe bacterial infection, but prolonged exposure of the neutrophils to such agents *in vivo*, may add to the killing defect observed *in vitro*.

Key words: Human neutrophils, endotoxin; bacterial antigens; function.

Chr. Koch, Blegdamshospitalet, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark.

Received 14.xi.74 Accepted 4.[illegible].74

Circulating neutrophil granulocytes from patients with bacterial infection display a number of functional alterations *in vitro*, including increased reduction of nitroblue-tetrazolium (NBT) (15, 16), indicating increased respiration (1), increased ingestion (11, 19), but markedly reduced intracellular killing of *Staphylococcus aureus* (11, 18). These changes are largely unexplained, but since increased NBT-reduction is easily induced *in vitro* by purified endotoxin (15) as

well as by antigenic products from different microbial species (8, 9, 14), it seemed of interest to study whether such products could also induce functional changes in ingestion and killing.

The present *experiments attempt to* answer these questions. Purified endotoxin, as well as a well-defined complex antigenic extract from *Pseudomonas aeruginosa,* were primarily studied, because the mechanism underlying induction of NBT-reduction by these agents has been extensively investigated (8, 9, 12, 15).

MATERIALS AND METHODS

Endotoxin as Bacto-Lipopolysaccharide *W, E. coli* 026:B6 and Bacto-Lipopolysaccharide *W, S. typhosa* 0901 were obtained from Difco Laboratories, Detroit, Mich. USA, and used freshly dissolved in 0.154 M saline. Water-soluble antigens from sonicated *Pseudomonas aeruginosa, Diplococcus pneumoniae, Neisseria meningitidis, Staphylococcus aureus,* without protein A, and *Streptococcus pyogenes* were kindly provided by Dr. *N. Høiby* of this department (Clin. Microbiol.). These extracts, containing multiple antigens, have been characterized in detail by *Høiby* & *Axelsen* (4) and *Høiby* (6). *Human serum albumin* (HSA) and rabbit anti-HSA antiserum were kindly donated by Dr. *J. M. Rhodes,* Statens Seruminstitut, Copenhagen. Precipitating HSA/anti-HSA complexes, *formed at* point of equivalence, and soluble complexes, formed at 8 times antigen-excess, were prepared as described previously (12). The serum used was either fresh serum from the donor of leucocytes = normal serum, or fresh serum from patients with chronic bronchitis, containing multiple precipitating antibodies against the *Ps. aeruginosa* antigens = immune serum, detected as described by *Høiby* (5).

Ingestion and intracellular killing of *Staph. aureus* were determined as described previously (11). Briefly, 2.5×10^6 polymorphonuclear leucocytes from normal adults were incubated at rotation with an approximately equal number of colony-forming units (CFU) bacteria in medium containing 10 per cent serum. Total CFU, intracellular CFU, and intracellular CFU in the presence of 10 mM sodium azide (NaN_3) which blocks killing (7, 10), were recorded at 1½ and 3 hours' incubation.

The various agents studied (endotoxin, antigens, and immune complexes) were either added directly to the leucocyte-bacteria reaction system at the start of incubation, or leucocytes were pre-incubated with the agent. Pre-incubation of leucocytes with endotoxin or *Ps. aeruginosa* antigens was performed in medium containing 10 per cent serum at 35° C for 20 minutes. Following a single centrifugation at 250 × *g* for 5 minutes, the cells were resuspended in fresh medium and serum, and bacteria added. *Pre-incubation* with *HSA/anti-*HSA complexes were, however, performed in medium without serum (to avoid albumin) ,adjusted to *physiological pH, followed by a single centri*fugation and resuspension in fresh medium, with serum and bacteria added as above.

Serum was thus present at a concentration of 10 per cent whether endotoxin or bacterial antigens were added directly to the system, or were pre-incubated with the cells.

The influence upon ingestion of the various agents is expressed as the ratio: intracellular CFU in NaN_3-treated cells in presence of the agent over simultaneously obtained control values in absence of the agent. The influence upon killing is expressed as the ratio: intra-cellular CFU in NaN_3-treated, divided by intracellular CFU in untreated cells, in the presence of the agent over simultaneously obtained control values in the absence of the agent.

NBT-tests were performed as described previously (8), after direct addition of agent to heparinized blood.

RESULTS

Tables 1 and 2 show the combined results which may be summarized as follows:

Stimulation of ingestion was seen after direct addition of endotoxin, of some antigens (*Ps. aeruginosa* and *D. pneumoniae*), of heat-killed *Staph. aureus* (boiled for 30 minutes), and after pre-incubation of the cells with precipitating and soluble immune complexes.

Inhibition of ingestion was seen after direct addition or pre-incubation with high concentrations (250 µg/ml) *of Ps. aeruginosa* antigens in the presence of immune serum, —but not in the presence of normal serum. A slight inhibition after pre-incubation with 1000 µg *E. coli* endotoxin per ml and after direct addition of 2 other bacterial *antigens* (*N. meningitidis* and *S. pyogenes*) was also *noted.*

Stimulation of killing was seen after direct addition of endotoxin, and of lower concentrations (0.009–0.9 µg/ml) of *Ps. aeruginosa* antigens, and after pre-incubation with soluble *immune complexes.*

TABLE 1. *Effects of Endotoxin and* Pseudomonas aeruginosa *Antigens upon Ingestion and Intracellular Killing of* Staphylococcus aureus *by Human Neutrophils* in vitro

Agent		Concentration µg per ml	Serum	Pre-incubation of leucocytes 35° C/20 min	Ingestion ratio* mean range	Killing ratio§ mean range	Per cent NBT positive neutrophils
E. coli endotoxin	(2)†	0.1	normal	—	1.59 (1.51–1.68)	1.60 (1.34–1.86)	29.5 (17.5)$
		10			1.42 (1.30–1.54)	1.44 (1.43–1.45)	51.0
		1000			1.12 (1.04–1.21)	1.11 (0.53–1.69)	79.5
S. typhosa endotoxin	(1)	0.1	normal	—	1.15	1.81	17.0 (5.0)
		10			1.26	1.98	36.0
		1000			1.30	1.06	68.0
E. coli endotoxin	(1)	0.1	normal	+	0.90	0.42	
		10			0.99	0.30	
		1000			0.73	0.26	
S. typhosa endotoxin	(1)	0.1	normal	+	1.12	0.42	21.0 (7.0)
		10			1.46	0.76	40.0
		1000			1.61	0.47	67.0
Ps. aeruginosa antigens	(3)	0 009	normal	—	1.03 (1.02–1.05)	1.25 (0.96–1.59)	16.0 (16.5)
		0.9			1.28 (1.21–1.37)	1.63 (1.26–1.91)	34.0
		90			1.14 (0.70–1.50)	0.30 (0.14–0.54)	62.0
Ps. aeruginosa antigens	(1)	*250*	normal	—	2.30	0.46	
Ps. aeruginosa antigens	(1)	*250*	immune	—	0.82	0.07	
Ps. aeruginosa antigens	(1)	*250*	normal	+	1.14	0.26	
Ps. aeruginosa antigens	(3)	*250*	immune	+	0.50 (0.29–0.68)	0.15 (0.05–0.31)	

* Ingestion Ratio is the number of intra-cellular colony-forming units (CFU) *Staph. aureus* after 3 hours incubation in sodium azide (NaN_3)-treated cells in the presence of agent over simultaneously obtained control values in the absence of agent.

§ Killing Ratio is the ratio: intracellular number of CFU after 3 hours in NaN_3-treated divided by intracellular CFU in untreated cells in the presence of agent over simultaneously obtained control values in the absence of agent.

Concentration is µg endotoxin or µg protein (microbial antigens) per ml leucocyte suspension (pre-incubated) or final leucocyte-bacteria suspension. Italicized concentrations: see Results.

† Number of experiments.

$ Unstimulated control values.

TABLE 2. *Effects of Various Microbial Antigens, Immune Complexes, and Heat-Killed* Staphylococcus aureus *upon Ingestion and Intracellular Killing of* Staphylococcus aureus *by Human Neutrophils* in Vitro

Agent		Concentration µg per ml	Serum	Pre-incubation of leucocytes 35° C/20 min	Ingestion ratio* mean range		Killing ratio§ mean range		Per cent NBT positive neutrophils	
D pneumoniae antigens	(1)†	5	normal	—	1.54		0.97		71.5	(8.0)$
N. meningitidis antigens	(1)	*83*	normal	—	0.78		0.28		83.0	(8.0)
Staph. aureus antigens	(1)	62	normal	—	0.97		0.39		61.5	(8.0)
S. pyogenes antigens	(1)	29	normal	—	0.88		0.71		38.0	(8.0)
Precipitating HSA/anti-HSA complexes	(3)	*33*[1]	—	+	1.75	(1.27–2.34)	0.74	(0.56–0.80)	34.0	(10.0)
HSA/anti-HSA complexes in 8 × antigen-excess	(2)		—	+	1.13	(1.03–1.24)	1.20	(0.94–1.45)	9.0	(10.0)
Heat-killed *Staph. aureus*	(1)	1:1[2]	normal	—	1.41		1.17			
		1:2.5			1.34		0.90			
		1:10			1.96		1.15			

For explanation: see legend for Table 1.
[1] µg HSA added to rabbit anti-HSA at point of equivalence.
[2] ratio: live to heat-killed (boiled for 30 minutes) bacteria.

tained control values in the absence of agent were also calculated. Expressed this way, the changes induced *in vitro* in the present studies did only in a few situations differ from the normal range of paired determinations in healthy persons (11). These situations,—italicized in Tables 1 and 2—were: pre-incubation with *E. coli* endotoxin at 10 and 1000 μg/ml and with *S. typhosa* endotoxin at 1000 μg/ml,—direct addition and pre-incubation with *Ps. aeruginosa* antigens at 90 μg/ml or above,—direct addition of *N. meningitidis* antigens,—pre-incubation with precipitating immune complexes,—and direct addition of heat-killed *Staph. aureus* at 2.5 times the number of live bacteria or more.

In neither situation were the changes, however, as pronounced as those seen in severely infected patients, as will be discussed below.

DISCUSSION

Ingestion is an active, energy-requiring process involving internalization of part of the membrane. The ingestion of many different microorganisms, including *Staph. aureus*, requires opsonizing serum factors for optimal ingestion. This is probably mediated through attachment of the Fc portion of IgG, or activated C3 to corresponding receptors on the neutrophils (13, 17). Simultaneously with, or shortly after ingestion, a rapid increase in oxydative metabolism occurs. This is a prerequisite for microbicidal activity (1), and it is quantitatively related to degree of particle ingestion.

The mechanism of initiation of increased respiration is largely unknown. It could be mediated through structural changes in the membrane, or directly triggered by attachment of opsonizing factors. Thus, degranulation and release of lysosomal enzymes, characteristic concequences of particle ingestion, may also be induced by attachment of the cells to non-phagocytosable surfaces coated with immune complexes (3).

NBT-reduction reflects the activity of oxydative metabolism (1) and stimulation of NBT-reduction can be induced by endotoxin. This stimulation depends upon the presence of specific antibodies and complement (15). Endotoxin produces a number of changes in the neutrophils, resembling those associated with phagocytosis (2). NBT-reduction can also be induced by complex microbial antigens (8, 9, 14) and recent findings show that, using various bacterial antigens, an active ingestion of antigen with participation of immunoglobulins and C3 occurs under experimental conditions that induce NBT-reduction (12).

In view of these different observations, it appears likely that the stimulation of ingestion, observed in these studies with endotoxin or *Ps. aeruginosa* antigens, represents increased membrane activity, secondary to ingestion of immune complexes (12), and further, that stimulation of killing is secondary to this membrane activation, either indirectly or directly through binding of opsonizing factors to corresponding surface receptors. This suggestion is strongly supported by the findings that heat-killed bacteria, as well as relatively simple precipitating HSA/anti-HSA complexes also induced increased ingestion, particularly since the latter have been shown to be actively ingested under these circumstances (12).

In several situations (large doses of microbial antigens, added directly or pre-incubated, and pre-incubation with precipitating immune complexes or endotoxin) a decrease in intracellular killing was noted. This appears not to depend upon suppression of respiration, since NBT-reduction was increased. More likely, this phenomenon represents wearing out of one or several factors involved in the killing process, or the direction of killing activity towards sites of the cell other than those containing the live bacteria. It was particularly pronounced using large doses of *Ps. aeruginosa* antigens in combination with immune serum; a situation, previously shown to induce high degree of stimulation of NBT-reduction, and ingestion of large amounts of antigen coupled to immunoglobulins and C3 (12). It should also be noted that rather modest concentrations of

gens in the presence or absence of human pseudomonas precipitins. Acta path. microbiol. scand. Sect. B, *81:* 787–794, 1973.

10. *Koch, C.:* Effect of sodium azide upon normal and pathological granulocyte function. Acta path. microbiol. scand. Sect. B, *82:* 136–142, 1974.
11. *Koch, C.:* Acquired defect in the bactericidal function of neutrophil granulocytes during bacterial infections. Acta path. microbiol. scand. Sect. B, *82:* 439–447, 1974.
12. *Koch, C., Høiby, N. & Wiik, A.:* Nitroblue-tetrazolium (NBT)-reduction by human peripheral neutrophil granulocytes in the presence of bacterial antigens. Evidence for immune complex ingestion. Acta path. microbiol. scand. Sect. C, *83:* 144–156, 1975.
13. *Lay, W. H. & Nussenzweig, V.:* Receptors for complement on leucocytes. J. Exp. Med. *128:* 991–1007, 1968.
14. *Matula, G. & Paterson, P. Y.:* Spontaneous in vitro reduction of nitroblue tetrazolium by neutrophils of adult patients with bacterial infection. New Engl. J. Med. *285:* 311–317, 1971.
15. *Nydegger, U. E., Miescher, A., Anner, R. M., Creighton, D. W., Lambert, P. H. & Miescher, P. A.:* Serum and cellular factor involvement in nitroblue tetrazolium (NBT) reduction by human neutrophils. Klin. Wschr. *51:* 377–382, 1973.
16. *Park, B. H., Fikrig, S. M. & Smithwick, E. M.:* Infection and nitroblue-tetrazolium reduction by neutrophils. Lancet *II:* 532–534, 1968.
17. *Quie, P. G., Messner, R. P. & Williams, R. C., Jr.:* Phagocytosis in subacute bacterial endocarditis. Localization of the primary opsonic site to the Fc fragment. J. Exp. Med. *128:* 553–570, 1968.
18. *Solberg, C. O. & Hellum, K. B.:* Neutrophil granulocyte function in bacterial infections. Lancet *II:* 727–729, 1972.
19. *Stossel, T. P.:* Evaluation of opsonic and leucocyte function with a spectrophotometric test in patients with infection and with phagocytic disorders. Blood *42:* 121–130, 1973.

Acta path. microbiol. scand. Sect. C, *83:* 203–209, 1975

DIFFERENT RATES OF DISAPPEARANCE OF COMMON ENTEROBACTERIAL ANTIGEN AND TYPE-SPECIFIC *E. COLI* O-ANTIGEN IN RAT PERITONEAL MACROPHAGES

OLE FROKJÆR THOMSEN and TAGE HJORT

University Institute of Pathology, Kommunehospitalet and Institute of Medical Microbiology, University of Århus, Denmark

Thomsen, O. Frokjær & Hjort, T. Different rates of disappearance of common enterobacterial antigen and type-specific *E. coli* O-antigen in rat peritoneal macrophages. Acta path. microbiol. scand. Sect. C, *83:* 203–209, 1975.

Common enterobacterial antigen (CA) and type-specific *E. coli* O-antigen were studied in rat peritoneal macrophages by means of immunofluorescence technique. At different time intervals after intraperitoneal injection of killed or viable *E. coli* O4, macrophages were harvested from the peritoneal fluid and the number of macrophages in which CA or *E. coli* O4-antigen, respectively, could be demonstrated was recorded. During the first few days, equal numbers of peritoneal macrophages were found to contain CA and *E. coli* O4-antigen, but on days 7–14 and onwards during the remaining experiment, i.e. throughout 8 weeks, CA was detected in fewer macrophages than *E. coli* O4-antigen, indicating a lesser degree of persistence of CA than of *E. coli* O4-antigen in the macrophages after phagocytosis of the bacteria. The results correspond with those previously obtained in experimental pyelonephritis in rats.

Key words: Rat peritoneal macrophages; enterobacterial antigen; *E. coli* O-antigen; disappearance.

O. Frokjær Thomsen, University Institute of Pathology, Kommunehospitalet, DK-8000 Århus C, Denmark.

Received 24.x 74 Accepted 10 xii.74

In kidney tissue with pyelonephritis, bacterial antigen has been demonstrated, using immunofluorescence technique, by means of antiserum against the specific O-antigen of the infecting strain, or antiserum against the common enterobacterial antigen (CA shared by nearly all strains of *E. coli* and most other Enterobacteriaceae (*Kunin et al.* 1962, *Kunin* 1963).

Regardless of which of these antisera is used, the diagnostic value of such observations must depend on the length of the period the bacterial antigen in question remains in the inflamed kidney tissue in such a state that it can be demonstrated.

In a study of experimental *E. coli* pyelonephritis in rats (*Thomsen & Hjort* 1973), CA was revealed in cases of acute pyelonephritis, but not in chronically inflamed kidney tissue in which, however, the specific bacterial O-antigen could be clearly demonstrated. A more detailed investigation of the matter (*Thomsen* 1974) revealed that, in

nearly all localizations, CA disappeared earlier from the inflamed kidney tissue than the type-specific O-antigen. The inflammation was characterized by the presence of numerous macrophages which seemed essential for the catabolization of the bacterial antigen. In these cells, type-specific O-antigen could be demonstrated for 14 weeks, CA for only 5 weeks.

As the macrophages seemed to be essential for the catabolization of the bacterial antigen, we desired to study the elimination of the two forms of antigen in these cells by means of a system particularly suitable for the observation of the function of macrophages. With this end in view, peritoneal macrophages were chosen and a modification of *Whitby & Rowley*'s method (1959) was used, according to which phagocytosis and growth of macrophages took place in the animal's own peritoneal cavity.

Bacteria were injected into the peritoneal cavity of rats, peritoneal fluid was removed at different time intervals, and the number of macrophages in which type-specific O-antigen and CA, respectively, could be demonstrated was determined by means of immunofluorescence technique.

MATERIAL AND METHODS

Bacteria. The injected bacterial strain was *E. coli* O4 U4/41. A suspension containing approx. 1×10^8 viable bacteria per ml was prepared as previously described (5). A portion of the suspension was heated at 100° C for 15 minutes in order to kill the bacteria.

Experimental procedure. White Wistar rats of both sexes, weighing 180–200 g, were used. Each rat received an intraperitoneal injection of 1 ml of the suspension of either killed or viable bacteria. Killed bacteria were used in order to study the time course of the disappearance of the antigen after injection of a given amount of antigen at a certain time (with no possibility of its increase in the organism). Viable bacteria were used in order to create a situation similar to that of a real infection, but it should be realized that, in these experiments, the amount of antigen and the time during which phagocytosis takes place may have varied considerably from animal to animal according to the bacterial proliferation in the organism. 40 rats received killed bacteria, and 28 received viable bacteria.

Sampling of peritoneal exudate. At different time intervals after the intraperitoneal injection, covering periods of up to 56 days after the injection, the animals were killed by an overdosis of ether. The peritoneal cavity was washed with 5 ml of sterile saline containing 5 IU heparin per ml. One ml of the fluid with the suspended peritoneal cells was withdrawn and placed on cleaned glass slides, each of which received 6 drops. The slides with the peritoneal washout were allowed to stand for 60 minutes at room temperature at which time the peritoneal macrophages were adhering to the glass. Then the supernatant fluid, with its content of other cells, was gently washed off and the smears were fixed in acetone for 10 minutes before immunofluorescence staining. Smears to be used for May-Grünwald-Giemsa staining were unfixed.

Immunofluorescence staining. The smears were washed in phosphate-buffered saline (pH 7.1). Indirect immunofluorescence staining was carried out as previously described for tissue sections (5). The first layer was an unlabelled rabbit antiserum against bacterial antigen, either anti-CA or anti-*E. coli* O4, and the second layer a commercially available antiserum against rabbit immunoglobulin (FITC-labelled goat anti-rabbit-γ-globulin) (Behringwerke).

Antisera. Antisera against *E. coli* O4 and CA were produced in rabbits by biweekly immunization with heat-killed *E. coli* O4 U4/41 and *E. coli* O14, respectively, as previously described (5). In order to exclude the possibility of a presence of antibodies against K-antigens, the antisera were tested by agglutination reactions with fresh suspensions (containing 0.5 per cent formalin) of *E. coli* O4 and *E. coli* O14, and with suspensions that had been boiled for two hours, so that only the O-antigen persisted. The reaction patterns obtained were exactly the same as those seen if reference O-antisera against *E. coli* O4 and *E. coli* O14, respectively, were used. Thus, the anti-O4 sera reacted with both fresh and boiled *E. coli* O4, indicating that our *E. coli* O4-strain possessed no K-antigen. The antisera against *E. coli* O14 (anti-CA) reacted with the boiled, but not with the fresh suspension of *E. coli* O14, indicating that the *E. coli* O14-strain contained a K-antigen, and that neither of the antisera contained detectable amounts of antibody against this antigen.

Moreover, in a number of control experiments, the immunofluorescence reactions obtained by our antisera did not differ from those obtained by the corresponding reference O-antisera.

Microscopic examination. A Zeiss universal fluorescence microscope with optical conditions as previously described was used (5). Fluorescent macrophages per 200 nucleated cells were counted.

TABLE 1. *Percentage of Fluorescent Macrophages/Number of Nucleated Cells in Smears Stained with Anti-O4 Serum and Anti-CA Serum*

Rats injected with killed *E. coli* O4						Rats injected with viable *E. coli* O4					
Day after inj.	% fluores. macr.		average %		diff. %	Day after inj.	% fluores. macr.		average %		diff. %
	anti-O4 ser.	anti-CA ser.	anti-O4 ser.	anti-CA ser.			anti-O4 ser.	anti-CA ser.	anti-O4 ser.	anti-CA ser.	
2	10	11									
2	10	8									
2	33	31									
2	6	6									
2	11	10									
3	30	29									
3	26	21				2	6	5			
3	17	20				2	3	3			
3	14	12				3	19	20			
3	4	6	16.1	15.4	0.7	3	12	10	10.0	9.5	0.5
6	29	25									
6	44	40									
7	21	20				6	27	27			
7	15	9				6	14	8			
7	8	0				6	13	16			
8	39	30				9	20	18			
8	30	19				9	15	4			
9	40	40	28.3	22.9	5.4	9	26	15	19.2	14.7	4.5
						13	21	8			
13	29	22				13	17	0			
13	24	18				13	0	0			
13	45	19				15	19	10			
15	37	27	33.8	21.5	12.3	15	28	16	17.0	6.8	10.2
20	44	35									
20	0	0				20	25	16			
20	9	0				20	28	17			
23	35	24	22.0	14.8	7.2	20	25	14	26.0	15.7	10.3
27	12	2									
27	0	0									
[illegible]	0	0				27	30	13			
[illegible]	30	18	10.5	5.0	5.5	27	17	1	23.5	7.0	16 5
34	10	2									
34	0	0				34	12	2			
34	0	0				34	19	1			
37	19	4	7.3	1.5	5.8	37	17	0	16.0	1.0	15 0
						41	19	0			
						41	39	2			
41	10	0				41	35	5			
44	18	5				44	4	0			
48	35	9	21.0	4.7	16.3	48	6	0	20.6	1.4	19.2
51	0	0									
56	15	0									
56	0	0	5.0	0.0	5.0						

cence, whereas the major part were without any fluorescence in the cytoplasm. However, some cells appeared with a more yellow fluorescence which was not localized in cytoplasmic droplets, but covered uniformly the entire cytoplasm (Fig. 1). Such cells were also seen in the control smears and this type of fluorescence was therefore considered to be non-specific. These cells, which seemed to be most predominant some time after injection of the bacteria, may represent degenerating or dead macrophages.

Controls. Specific fluorescence was present only in macrophages stained with homologous antiserum or anti-CA serum as the first layer. All other controls did not show specific fluorescence.

Differential counts. All cells with cytoplasmic fluorescent droplets were recorded as macrophages with phagocytized bacterial antigen and determined as a percentage of the total number of nucleated cells.

Table 1 shows the percentage of macrophages stained with anti-O4 and anti-CA serum, respectively, in each animal. The animals were examined at certain intervals after injection of bacteria (on days 2–3, and about 1, 2, 3, 4, 5, 6, 7 and 8 weeks after the injection). For each of the subgroups, the average percentage has been calculated and listed in the table. It appears that, after injection of either killed or viable bacteria, the percentage values varied considerably from animal to animal within the various subgroups, particularly late in the experiment when some animals revealed zero values while others still presented fairly high numbers of stained macrophages. Furthermore, late in the experiment, the subgroups are rather small, and the average values therefore subject to a great uncertainty.

However, this does not influence the possibility of comparing the number of O4-antigen- and CA-containing macrophages in the individual animals; such comparison reveals a definite pattern: On days 2–3, all animals had practically identical percentage values of O4-antigen- and CA-containing macrophages, whereas the remaining experimental period was characterized by percentage values of macrophages containing O4-antigen consistently higher than percentage values of CA-containing macrophages. Thus, about one week after injection, this difference averaged 5.4 per cent for killed and 4.5 per cent for viable bacteria and, after about 2 weeks, the difference was still more pronounced, viz 12.3 per cent for killed, 10.2 per cent fo viable bacteria. It should be added that, cor currently with the decreasing number of flu rescent macrophages, fluorescent cytoplasm droplets were generally fewer and weaker anti-CA stains than in anti-O4 stains.

The diagram in Fig. 3 illustrates changes in the average percentage values -- O4-antigen- and CA-containing macrophages, calculated for the individual subgroups. After administration of killed bacteria, the maximum number of fluorescent cells was reached fairly early in the experiment, after one week in the case of CA-containing cells and after two weeks in the case of O4-antigen-containing cells. From day 14, both curves reveal a fall, reaching a plateau in the fourth week (the high average percentage of macrophages stained with anti-O4 serum 6–7 weeks after the injection of killed bacteria is due to one single animal in which the value was extraordinarily high). In the animals receiving viable bacteria there was a more gradual increase in the number of cells stained with anti-O4 serum. The maximum was seen after 3–4 weeks and the level remained fairly high throughout the experiment. The percentage of macrophages with CA was found to be essentially unchanged during the first 4 weeks upon which it decreased to negligible levels.

DISCUSSION

The technique used in the present investigation offers several advantages, being easy to work with and yielding good visualization of the antigens. The disadvantage is that it does not involve a fixed number of macrophages, as in *in vitro* experiments, but an interchangeable pool of macrophages. Thus, antigen-containing macrophages may have disap-

Acta path. microbiol. scand. Sect. C, *83:* 210–214, 1975

INFLUENCE OF METHYLPREDNISOLONE AND AZATHIOPRINE ON POLYMORPHONUCLEAR NEUTROPHILS (PMN) AND LYMPHOCYTES IN GERMFREE, MONOCONTAMINATED AND CONVENTIONAL RATS

A. Baardsen, T. Midtvedt and A. Trippestad

Kaptein Wilhelm Wilhelmsen og Frues Bakteriologiske Institutt, University Hospital, Rikshospitalet, Oslo, Norway

Baardsen, A., Midtvedt, T. & Trippestad, A. Influence of methylprednisolone and azathioprine on polymorphonuclear neutrophils (PMN) and lymphocytes in germfree, monocontaminated and conventional rats. Acta path. microbiol. scand. Sect. C, *83:* 210–214, 1975.

In rats, protracted administration of methylprednisolone at high dose levels 1) increased the number of circulating PMN in germfree, monocontaminated (with *Escherichia coli*) and conventional rats. This drug-induced increase was more than doubled in the presence of microorganisms, as compared to germfree rats. 2) reduced the number of circulating lymphocytes more strongly in germfree rats than in monocontaminated and conventional rats. Protracted administration of azathioprine at high dose levels 1) reduced the number of circulating PMN in conventional rats, but not in germfree and monocontaminated rats. 2) did not affect the lymphocyte count at the dose level 40 mg per kg per day in any of the three groups of animals investigated.

Key words: methylprednisolone; azathioprine; leucocytes; germfree life.

A. Baardsen, Kaptein Wilhelm Wilhelmsen og Frues Bakteriologiske Institutt, University Hospital, Rikshospitalet, Oslo, Norway.

Received 12.xii 74 Accepted 12.xii.74

Alterations in the number of circulating leucocytes observed during treatment with immunosuppressive drugs, such as glucocorticosteroids (steroids) and azathioprine (AZA), might partly be explained as a primary drug-induced effect and partly as a secondary effect due to microorganisms present.

Separation of primary drug effects from alterations induced by microorganisms is difficult in conventional individuals living in an uncontrolled microbiological environment (7).

In germfree (GF) animals the microbial factor is eliminated. The leucocyte counts in these animals might be termed "basic counts". These animals seem well suited for the evaluation of drug-induced influence on the number of circulating leucocytes. The

addition of one known strain of bacteria to GF animals might provide a model for evaluation of the additional haematological alterations induced by the presence of bacteria during treatment with immunosuppressive drugs.

The aim of the present study on GF, monocontaminated and conventional rats has been to evaluate the proper drug-induced effect on the number of circulating PMN and lymphocytes, and to separate this effect from that induced by bacteria during treatment with methylprednisolone (MP) or AZA.

MATERIALS AND METHODS

Animals

All rats used in the present study were of the CDF strain (Charles River Breeding Labs., Wilmington, Mass., USA). They were used without regard to sex and their age varied from 2–4 months. *GF rats* were reared as previously described (1). *Monocontaminated rats* were obtained by contaminating GF rats with the same strain (X7) of *Escherichia coli* as previously used (1, 2). *Conventional rats* of the CDF strain, conventionalized for more than 10 generations, were used.

The rats were grouped according to treatment as listed in Table 1.

Drug Treatment

Methylprednisolone (MP)-succinate in aqueous solution (Solu-Medrol®, The Upjohn Company, Kalamazoo, Mich., USA) was given s.c. in daily doses of 40 mg per kg for 3 weeks to groups of conventional rats. The corresponding groups of GF and monocontaminated rats were treated s.c. with equivalent doses of MP-hemisuccinate (Urbason solubile®, Farbwerke Hoechst, Frankfurt (M), Germany) from sterile ampules (2). Treatment of monocontaminated rats was initiated simultaneously with contamination, except in one group of rats treated with MP 50–100 mg per kg per day. In the latter group, 6 mg MP was injected per rat at hour −24, −12 and −1 prior to contamination, and thereafter once daily for a further 12 days (2).

AZA (Imuran®, Burroughs Wellcome & Co) in aqueous solution was injected intraperitoneally once daily for 21 days in conventional rats and for 14 days in GF and monocontaminated rats, as previously described (1). A standard dose of 40 mg per kg per day was used in most rats. One group of conventional rats was treated with AZA 60 mg per kg per day

Blood Samples

were collected from the tail vein under general anaesthesia with ether-ethanol 1:1 (v/v), on the day after termination of drug treatment and, in conventional rats, also immediately prior to treatment.

Leucocyte Counting

was performed using an electronic particle counter (Celloscope 101, AB Lars Ljungberg & Co., Stockholm, Sweden) as described by *Kvarstein* (9). The 20 μl blood sample was diluted in 2ml of 0.15 M NaCl before addition of the haemolysing diluent.

Differential Leucocyte Count

Blood smears were stained with May-Grünwald-Giemsa and at least 100 cells were counted per sample.

Statistical Analysis

The two-samples ranks test of Wilcoxon-Whi[illegible] was used for statistical analysis (10).

RESULTS

All rats survived treatment with MP or AZA at the dose level 40 mg per kg per day, while AZA 60 mg per kg per day was lethal to 2 of 6 conventional rats treated for 3 weeks. The highest dosages of either drug resulted in severe wasting of the rats.

The results are shown in Table 1.

White Blood Cell Counts (WBC), cells/mm³

a) Controls. In GF and monocontaminated rats, the mean WBC were of the same order of magnitude. In conventional controls, WBC were higher, up to twice that of GF controls.

b) Treated groups. Treatment with MP or AZA did not significantly ($p>0.10$) reduce WBC below the level of GF and monocontaminated controls, except in the group of conventional rats treated with AZA 60 mg per kg per day. WBC supplemented with differential counts of leucocytes indicated drug effects on the number of circulating PMN and lymphocytes, as described in detail below.

TABLE 1. *Total White Blood Cell Counts (WBC) and Number of PMN and Lymphocytes per mm³ Blood from Germfree (GF), Monocontaminated (MONO) and Conventional (CONV) Rats. Influence of Treatment with Methylprednisolone (MP) or Azathioprine (AZA)*

State	Treatment	Days*	WBC ± SEM/n	PMN ± SEM/n	Lymphocytes ± SEM/n
GF	Controls	14	6042 ± 457/12	1304 ± 190/12	4534 ± 301/12
	MP (40)	14	4325 ± 359/ 8	2720 ± 320/ 6	1502 ± 325/ 6
	AZA (40)	14	6467 ± 1181/ 3	1569 ± 140/ 3	4694 ± 1261/ 3
MONO	Controls	14	5410 ± 390/20	1277 ± 128/20	3994 ± 399/20
	MP (40)	14	5742 ± 709/12	3558 ± 273/12	2010 ± 525/12
	MP§ (50–100)	14	6600 , 7500/ 2	5940 , 6750/ 2	0 , 300/ 2
	AZA (40)	14	4317 ± 916/ 6	1136 ± 354+6	3123 ± 559/ 6
CONV	Controls	0	9836 ± 804/14	1525 ± 222/14	8067 ± 662/14
	Controls	21	7707 ± 554/14	2616 ± 350/14	5664 ± 369/15
	MP (40)	0	9414 ± 840/14	1486 ± 173/13	7363 ± 709/13
	MP (40)	21	7123 ± 429/13	3586 ± 235/13	3266 ± 306/13
	AZA (40)	0	10400 ± 944/14	1348 ± 193/14	8690 ± 900/14
	AZA (40)	21	5817 ± 462/12	571 ± 120/12	5151 ± 400/12
	AZA (60)	0	9183 ± 455/ 6	1548 ± 54/ 6	7398 ± 395/ 6
	AZA (60)	21	3733 ± 763/ 3	172 ± 66/ 3	3489 ± 735/ 3

* Days of treatment; in controls days of observation.
§ See Materials and Methods. Data given are individual observations.
SEM = standard error of the mean. n = number of observations. Brackets indicate dose of drug (mg per kg per day).

Number of PMN/mm³

a) Controls. In GF and monocontaminated rats, the mean number of PMN approximated 1300. The corresponding mean value of conventional control rats varied up to twice that of GF rats during the observation period.

b) MP-treatment of GF rats (40 mg per kg) resulted in approximately 100 per cent increase in the number of circulating PMN in relation to untreated controls. In monocontaminated and conventional rats, the same dose of MP resulted in a number of PMN approximating 3600 which is nearly three times that of GF controls. The highest number of PMN was recorded in the monocontaminated rats treated with MP (50–100 mg per kg) from one day prior to contamination.

c) AZA-treatment of GF rats (40 mg per kg) and monocontaminated rats did not alter the number of PMN significantly compared to that of untreated controls ($p>0.10$). In conventional rats, however, the same treatment reduced the number of PMN to less than 50 per cent of the initial values. In conventional rats treated with AZA 60 mg per kg, the number of PMN was reduced to as little as 10 per cent of the pretreatment values.

Number of Lymphocytes/mm³

a) Controls. The mean number approximated 4000 in GF and monocontaminated control rats. The corresponding number in conventional controls varied from 5600 to 8000 during the 3 weeks' observation period.

b) MP-treatment induced a significant ($p<0.01$) reduction in the number of circulating lymphocytes in GF, monocontaminated and conventional rats. This reduction was most marked in GF rats, showing a lymphocyte count which was 33 per cent in relation to that of untreated controls. The group of monocontaminated rats pre-treated with MP prior to contamination and further treated with MP 50–100 mg per kg day, showed an almost complete absence of circulating lymphocytes.

c) AZA-treatment did not significantly reduce the number of circulating lymphocytes in GF and monocontaminated rats ($p>0.10$). Considering the variation in number of lymphocytes in untreated conventional controls, the standard dose (40 mg per kg) did not significantly reduce the lymphocyte counts in conventional rats while the higher dose (60 mg per kg) induced a 50 per cent reduction in the number of circulating lymphocytes during the period of treatment.

DISCUSSION

The observation that the number of circulating PMN in GF rats was doubled by steroid treatment suggests a primary steroid effect on the production or mobilization of PMN. A similar phenomenon has been observed in mice (4). At the same dose level, the PMN count in conventionalized rats and in the simultaneously monocontaminated rats increased up to three times that in GF controls. This additional increase in PMN count was probably due to the presence of microorganisms, as demonstrated in mice by others (4, 11). Rats exposed to the highest dose of MP, from one day (in GF state) prior to contamination, showed the highest PMN count. It appears that the latter is additionally increased by pre-treatment with MP; either the primary drug effect or the host reaction to a second factor, the bacterial stimulus, is altered. The primary drug effect is suggested to be equal in GF and contaminated rats, and consequently independent on timing of steroid administration in relation to monocontamination. It seems more likely that pretreatment alters the relationship between host and bacteria. The phagocytic function of the reticulo-endothelial system is regularly depressed in GF animals (3). In addition, steroids induce an extreme lymphoid atrophy in these animals (*vide infra*). It might therefore be expected that GF rats pre-treated with MP are less resistant to bacteria than GF rats not pre-treated. Consequently a higher bacterial influence on production or mobilization of PMN might be anticipated in the former group of rats.

It follows from the present results that the number of circulating lymphocytes was reduced in all rats treated with MP. At equal dose level, this reduction was most marked in GF rats. Steroid-induced lymphocytopenia has been explained as a result of lymphocytolysis and lymphoid atrophy (6, 12). The lymphoid tissues of GF animals are characterized by a scarcity of cells, reflecting the lack of antigenic stimulus in these animals (5). As the maturation of lymphocytes depends on the functional state of the lymphoid tissues, it might therefore be expected that the steroid-induced lymphocytopenia would be more marked in GF rats than in contaminated rats. The extreme lymphocyte depletion observed in the particular group of GF rats treated with MP prior to contamination, might in part be dose-dependent. However, as mentioned above, these rats received MP during one day (in GF-state) prior to the introduction of *E. coli*. Their lymphocyte depletion might therefore correspond to that of steroid-treated GF rats.

It has been shown by others that administration of AZA to man (8) and rodents (8) reduces the number of circulating PMN, probably by depressing their formation. Whether AZA affects the circulating cells directly is still a matter of dispute.

Our results confirm the neutropenic effect of AZA in conventional rats. It is an interesting observation that the same effect of AZA could not be demonstrated in the GF and monocontaminated series of rats. If the influence of AZA depends on metabolism to active components, it might be that this metabolic rate is lower in GF and monocontaminated rats than in conventional rats. In other words, the metabolism or enzymatic activation of AZA seems to be conditioned by the presence of microorganisms other than *E. coli*. It seems unlikely that the lack of neutropenic effect of administration of AZA for two weeks to GF and monocontaminated rats should be related to differences in the turnover rate of PMN.

AZA in doses smaller than 60 mg per kg did not reduce the number of circulating lymphocytes in either series of rats. That is in harmony with the results of *Gotjamanos* (8) who found that a significant reduction in size of lymph nodes and cellular depletion of lymphatic nodules in mice was evident only at the highest dose levels (75 mg per kg).

Increased susceptibility to infections following administration of MP is a well-established fact. As shown, this cannot be explained by an influence of MP on the number of circulating PMN, while the lymphopenic effect might be of importance. AZA might, however, enhance infection by reducing the PMN count. The same drug seems to affect the lymphocyte count only when administered in sublethal doses.

REFERENCES

1. *Baardsen, A., Midtvedt, T. & Trippestad, A.:* Influence of azathioprine on humoral defence factors against *Escherichia coli* in germfree and monocontaminated rats. Acta path. microbiol. scand. Sect. B, *81:* 799–805, 1973.
2. *Baardsen, A., Midtvedt, T. & Trippestad, A.:* Influence of methylprednisolone on complement activity in germfree rats and on antibody activity to *Escherichia coli* in monocontaminated rats. Acta path. microbiol. scand. Sect. B, *83:* 37–44, 1975.
3. *Benveniste, J., Higounet, F. & Salomon, J.-C.:* Effects of various doses of prednisolone on the phagocytic activity in axenic and holoxenic mice. RES, J. Reticuloendothelial Soc. *8:* 499–507, 1970.
4. *Branceni, D., Benveniste, J. & Salomon, J.-C.:* Effets de l'administration continue massive d'un glucocorticoïde chez la souris axenique. Études anatomo-pathologiques, hématologiques et biochimiques. Ann. Inst. Pasteur *114:* 828–845, 1968.
5. *Crabbé, P. A., Nash, D. R., Bazin, H., Eyssen, H. & Heremans, J. F.:* Immunohistochemical observations on lymphoid tissues from conventional and germ-free mice. Lab. Invest. *22:* 448–457, 1970.
6. *Dustmann, H. O. & Stolpmann, H. J.:* Die Beeinflussung des Lympknotens durch Glucocorticosteroid. Virchows Arch. Abt. A, Path. Anat. *345:* 121–131, 1968.
7. *Godwin, K. O., Fraser, F. J. & Ibotson, R. N.:* Haematological observations on healthy (SPF) rats. Brit. J. exp. Path. *45:* 514–524, 1964.
8. *Gotjamanos, T.:* The effect of azathioprine on phagocytic activity and morphology of reticuloendothelial organs in mice. Pathology *3:* 171–179, 1971.
9. *Kvarstein, B.:* The use of an electronic particle counter (Celloscope 101) for counting leucocytes. Scand. J. clin. Lab. Invest. *19:* 196–202, 1967.
10. *Mainland, D.:* Elementary medical statistics. 2. ed. W. B. Sounders Company, Philadelphia and London 1964.
11. *Reed, N. D. & Jutila, J. W.:* Wasting disease induced with cortisol acetate I. Studies in germ-free and conventionally reared mice. J. Immun. *99:* 238–245, 1967.
12. *Videbæk, Aa.:* Glucocorticoids in haematology. Acta med. scand. Suppl. *500:* 35–41, 1969.

MATERIALS AND METHODS

Sample Collection and Purification Procedure

Whole saliva was collected from donors, secretors and non-secretors, of blood groups A and B. Secretory rate was stimulated by wax chewing, and the saliva was collected in ice-chilled, teflon-coated glass tubes.

Purification of the salivary blood-group substance was done by elimination of heat labile proteins and by chromatography on Bio-Gel P 300 as described previously (*Rölla* & *Jonsen* 1968).

Hydrolysis and Amino Acid Analysis

Samples for hydrolysis were transferred to freeze-dry ampoules, evaporated to dryness and suspended in 2–3 ml 6 N HCl. The ampoules were then evacuated and sealed under vacuum as previously described (*Sönju* & *Rölla* 1971). Hydrolysis was done at 105° C for 24, 48 and 72 hours. The hydrolysates were evaporated to dryness and redissolved in a 0.2 M sodium acetate buffer pH 2.8. Norleucine, 0.1 μ mol, was added as internal standard immediately before analysis.

Amino acid analysis was performed with a Technicon AutoAnalyzer using the 18-hour standard elution procedure with Chromobead Type B resin. Qualitative determinations were done by estimation of elution positions and by enrichment of the hydrolysates with individual amino acids. A Technicon Integrator/Calculator Model AAG was used for quantitative determinations.

Colour factors were obtained from analysis of a standard amino acid solution (Technicon Standard Amino Acid Solution, AR 112-62 [807 F]), containing 0.1 μ mol of asp, thr, ser, glu, pro, gly, ala, val, cys, met, ile, leu, tyr, phe, lys, his, arg. The standard solution was enriched with 0.1 μ mol of cysteic acid, glucosamine and galactosamine.

Alkali Treatment

Blood-group active substance was purified from whole saliva of a donor of secretor status of blood group O. Material from several preparations was pooled and dialysed against distilled water for 72 hours. The dialysate was then evaporated and made up to 10 ml with distilled water. The amount of material in 1 ml of solution was determined after drying to constant weight at 60° C.

A solution of 0.3 M sodium-borohydride in 0.1 N NaOH, containing 1 mg glycoprotein per ml, was then prepared and stored at 4° C. One sample (T_0), containing 1 mg glycoprotein, was immediately neutralized with N acetic acid. One drop of octanol was added to prevent excess foaming, and the solution was dialysed against distilled water for 48 hours. Aliquots were also taken after 72 and 192 hours.

Release of the carbohydrate side chain of the salivary glycoprotein would presumably cause loss of virus haemagglutination inhibition activity, as this activity is related to the presence of sialic acid in a terminal position of the carbohydrate side chain (*Gottschalk et al.* 1972a). The alkali treated and dialysed aliquots were therefore tested for changes in haemagglutination inhibition activity according to *Francis* & *Minuse* (1948), using B/Lee influenza virus in the indicator state.

As a control of the alkali treatment, Bovine Submaxillary Mucin (BSM) (Boehringer, Mannheim) was subjected to the same treatment as the human salivary preparation with samples taken at T_0 and T_{192} hours.

Sulphate Analysis

Sulphate determinations were performed according to *Terho* & *Hartiala* (1971), using sodium rhodizonate and measuring the reduction in optical density at 520 nm caused by the sulphate after the addition of barium to give a red colour.

RESULTS

Amino Acid Analysis

The amounts of the individual amino acids as shown in Fig. 1 are calculated from the mean of the amounts found at the various times of hydrolysis, except for the hydroxy- and sulphur-containing amino acids where the highest amounts are shown. Only the composition of the secretor material is shown as no difference between the secretor and non-secretor material could be detected. The amino acid composition of blood-group O material (*Sönju & Rölla 1971*) is included for comparison.

The most characteristic features of the amino acid composition were large amounts of acidic and neutral amino acids and small amounts of sulphur-containing and basic amino acids. The high amounts of acidic and neutral amino acids, together with the small amounts of sulphur-containing amino acids and histidine, give the glycoprotein a characteristic amino acid profile.

The amounts of the amino sugars varied considerably (Table 1) and no definite pattern was evident, except that all the preparations contained more glucosamine than galactosamine and that the non-secretor glyco-

protein of blood-group O status (from *Sönju* & *Rölla* 1971) contained 4–7 times as much glucosamine as any of the other preparations.

The differences between the present salivary material and the cystic material presented by *Pusztai* & *Morgan* (1963) are demonstrated in Fig. 2. It is of particular interest that the cystic material contains abundant amounts of threonine and serine, whereas the salivary material contains relatively large amounts of aspartic acid.

Alkali Treatment

Amino acid analysis of the human salivary preparation removed after 72 and 192 hours showed no difference in the amounts of threonine, serine, glucosamine, and galactosamine as compared with the T_0 sample (Table 2).

TABLE 2. *Recovery of Hydroxy Amino Acids and Hexosamines after Alkali Treatment. µg Amino Acids and Hexosamines/mg Glycoprotein*

	T_0	T_{72}	T_{192}
Human salivary glycoproteins			
Threonine	20.00	21.00	21.00
Serine	17.00	18.00	18.00
Glucosamine	0.04	0.03	0.03
Galactosamine	0.02	0.02	0.02
Bovine submaxillary glycoprotein			
Threonine	23.00		9.2
Serine	34.0		12.4
Glucosamine	0.3		–
Galactosamine	2.0		–

A slight decrease in the virus haemagglutination inhibition titre was found after 192 hours of alkali treatment, whereas no effect was found after 72 hours.

Analysis of BSM subjected to the same treatment as the human salivary glycoprotein gave a reduction of 60 per cent in the amount of threonine, and 65 per cent in the amount of serine after 192 hours. The amounts of glucosamine and galactosamine were too small to obtain reliable estimates after 192 hours of alkali treatment.

Sulphate Analysis

The results of the sulphate determinations showed that the human salivary glycoprotein from secretors of blood groups A, B and O all contained a substantial amount of sulphate (Table 3).

TABLE 3. *Sulphate Content of a Sublingual Glycoprotein Isolated from Individuals Possessing Different Blood-Group Status*

	Secretor		
	A	B	O
Sulphate µg/mg glycoprotein	5.6	5.9	3.7

The mean of two analyses from each blood group is shown.

DISCUSSION

The investigated substance is homogeneous in the ultracentrifuge and by disc electrophoresis (*Rölla* & *Jonsen* 1968). The finding that the blood-group substance from saliva of secretors contains twice the amount of fucose as the corresponding preparation from non-secretors (*Sönju* & *Rölla* 1971) is in accordance with the concept that the protein specified by the secretor gene is an α-2-L-fucosyl transferase (*Watkins* 1971) and may therefore be taken as a further indication of a homogeneous preparation.

The glycoprotein possesses virus haemagglutination inhibition and blood-group substance activity and has a high affinity for hydroxyapatite (*Rölla* & *Mathiesen* 1970). The affinity for hydroxyapatite is consistent with the acidic characteristics of the glycoprotein (*Bernardi* & *Kawasaki* 1968).

The amino acid analyses indicated a fairly similar amino acid composition of glycoproteins isolated from donors of blood groups A and B, and the composition did not vary from the previously investigated glycoprotein of blood group O activity (*Sönju* & *Rölla* 1971). The small differences observed may be caused by minor variations in the experimental procedures or by the presence of small amounts of contaminants. The pre-

sence of carbohydrates has been reported to interfere with amino acid analyses (*Gottschalk* 1972) and may also cause apparent variations between different blood groups.

Pusztai & *Morgan* (1963) investigated the amino acid composition of ovarian cyst material and found that the composition is closely similar irrespective of the serological specificity within the ABO and Lewis systems. However, this view is not shared by *Donald* (1973) who found that purified blood-group substance from ovarian cysts has different amino acid composition for the various blood groups, but leaves cores of very similar amino acid composition after pronase treatment.

Amino acid analysis of the erythrocyte antigens (*Gardas* & *Kościelak* 1973) indicates similarity between the amino acid compositions of the A, B and H-active substances. The A-active substance, however, contains more leucine and glutamic acid than the B and H-active, but the differences are probably not significant.

Definite conclusions as to the similarity of the amino acid compositions of the various blood groups of human salivary origin should therefore be postponed until more information has been gained. However, our results indicate a similar amino acid composition of the various human salivary blood-group specific glycoproteins.

The estimation of amino sugars after acid hydrolysis intended for the hydrolysis of peptide bonds is subjected to a number of uncertainties. The hexosamines are more stable to acid than most of the monosaccharides, but they are less stable than the majority of amino acids. The rate of destruction is also dependent on the presence of oxygen (reviewed by *Neuberger et al.* 1966), so that small variations in the evacuation before hydrolysis may cause great variations in the estimated amounts. The colour yield with ninhydrin is also poorer for hexosamines than for most of the amino acids, and since the relative amounts of the hexosamines in the salivary glycoprotein are small, the figures in Table 1 should be regarded as preliminary.

It is interesting to note that the human salivary preparation has an amino acid composition very similar to the A, B and H erythrocyte antigens isolated by *Gardas* & *Kościelak* (1973). Their preparation contained about the same relative amounts of aspartic and glutamic acids, i.e. about the same amount as that of threonine and serine, and small amounts of histidine. In contrast, the differences between the ovarian cyst material and the salivary preparations are evident (Fig. 2).

The difference is further illustrated by the observation that the ovarian cyst material is alkali labile (*Donald* 1973). The alkali stability of our material is also in contrast to the BSM found to be alkali labile both in the present control experiments and in previous investigations (reviewed by *Gottschalk et al.* 1972b). The alkali labile *O*-glycosidic bond present in ovarian cyst material and in BSM involves the hydroxyl group of threonine and serine. It may be relevant to this discussion that the amount of threonine and serine is significantly greater in the alkali labile ovarian cyst material and BSM than in the alkali stable salivary and erythrocyte preparations. On this background, few arguments point to the *O*-glycosidic type of carbohydrate protein linkages in the salivary preparation, leaving greater possibility for the *N*-acetyl glucosamine-asparagine type.

The glycoprotein analysed in the present investigation originates from the sublingual saliva (*Rölla* 1967) and the presence of sulphomucins in the sublingual gland has been established histochemically (*Eversole* 1972). The presence of sulphate in the present preparation was therefore not surprising, especially when considering the low isoelectric point of the glycoprotein (*Rölla* & *Jonsen* 1968). The amounts of sulphate varied somewhat between the various blood groups, but the variations were probably caused by difficulties in estimating the dry weight of the glycoprotein because of hygroscopic properties of the material.

REFERENCES

Bernardi, G. & *Kawasaki, T.*: Chromatography of polypeptides and proteins on hydroxyapatite columns. Biochim. biophys. Acta *160:* 301–310, 1968.

Donald, A. S. R.: The products of pronase digestion of purified blood group-specific glycoproteins. Biochim. biophys. Acta *317:* 420–436, 1973.

Eversole, L. R.: The mucoprotein histochemistry of human mucous acinar cell containing salivary glands: Submandibular and sublingual glands. Arch. oral Biol. *17:* 43–53, 1972.

Francis, T., Jr. & *Minuse, E.*: Influence of saliva upon hemagglutination by influenza virus. Proc. Soc. exp. Biol. (N.Y.) *69:* 291–294, 1948.

Gardas, A. & *Kościelak, J.*: New form of A-, B-, and H-blood-group-active substances extracted from erythrocyte membranes. Europ. J. Biochem. *32:* 178–187, 1973.

Gottschalk, A.: Interaction between reducing sugars and amino acids under neutral and acidic conditions. In: Gottschalk, A. (Ed.): Glycoproteins. Their composition, structure and function. Revised and expanded second edition. B.B A. Library, Vol. 5. Elsevier, Amsterdam/London/New York 1972, pp. 141–157.

Gottschalk, A., Belyavin, G. & *Biddle, F.*: Glycoproteins as influenza virus haemagglutinin inhibitors and as cellular virus receptors. In: Gottschalk, A. (Ed.): Glycoproteins. Their composition, structure and function. Revised and expanded second edition. B.B.A. Library, Vol. 5. Elsevier, Amsterdam/London/New York 1972a, pp. 1082–1096.

Gottschalk, A., Bhargava, A. S. & *Murty, V. L. N.*: Submaxillary gland glycoproteins. In: Gottschalk, A. (Ed.): Glycoproteins. Their composition, structure and function. Revised and expanded second edition. B.B.A. Library, Vol. 5. Elsevier, Amsterdam/London/New York 1972b, pp 810–829.

Kabat, E A, Bendich, A., Bezer, A. E. & *Beiser, S M*: Immunochemical studies on blood groups. IV. Preparation of blood group A substance from human sources and comparison of their chemical and immunochemical properties with those of the blood group A substance from hog stomach. J. exp. Med. *85:* 685–699, 1947.

Martin, F., Mathian, R., Berard, A. & *Lambert, R.*: Sulfated glycoproteins in human salivary and gastric secretions. Digestion *2:* 103–112, 1969.

Morgan, W. T. J. & *van Heyningen, R.*: The occurrence of A, B and O blood group substances in pseudo-mucinous ovarian cyst fluids. Brit. J. exp. Path. *25:* 5–15, 1944.

Neuberger, A., Marshall, R. D. & *Gottschalk, A.*: Some aspects of the chemistry of the component sugars of glycoproteins. In: Gottschalk, A. (Ed.): Glycoproteins. Their composition, structure and function. B.B.A. Library, Vol. 5. Elsevier, Amsterdam/London/New York 1966, pp 158–189.

Pusztai, A. & *Morgan, W. T. J.*: Studies in immunochemistry. 22. The amino acid composition of the human blood-group A, B, H and Le^a specific substances. Biochem. J. *88:* 546–555, 1963.

Rölla, G.: A calcium-binding glycoprotein from human saliva. Thesis. Universitetsforlaget, Oslo 1967.

Rölla, G. & *Jonsen, J.*: A glycoprotein component from human sublingual-submaxillary saliva. Caries Res. *2:* 306–316, 1968.

Rölla, G. & *Mathiesen, P.*: The adsorption of salivary proteins and dextrans to hydroxylapatite. In: McHugh, W. D. (Ed.): *Dental plaque.* Livingstone, Edinburgh 1970, pp. 129–142.

Schrager, J. & *Oates, M. D. G.*: Further studies of the principal glycoprotein from human saliva. Arch. oral Biol. *16:* 1269–1279, 1971.

Sönju, T. & *Rölla, G.*: Chemical analysis of a salivary glycoprotein with blood-group substance and virus inhibition activities. Acta path. microbiol. scand. Sect. B, *79:* 95–101, 1971.

Terho, T. T. & *Hartiala, K.*: Method for determination of the sulfate content of glycosaminoglycans. Analyt. Biochem. *41:* 471–476, 1971.

Watkins, W. M.: Pathways towards the ABH antigens of red cells and secretions. Brit. J. Haemat. *21:* 352 (only), 1971.

Acta path. microbiol. scand. Sect. C, *83:* 221–230, 1975

EXPERIMENTAL STUDIES ON CHOLERA IMMUNIZATION

V. Cross-reactive and Typespecific Determinants on V. cholerae *Inaba and Ogawa Endotoxin and Their Significance for Protective Immunity*

A.-M. Svennerholm, J. Holmgren and Ö. Ouchterlony

Institute of Medical Microbiology, University of Göteborg, Göteborg, Sweden

Svennerholm, A.-M., Holmgren, J. & Ouchterlony, Ö. Experimental studies on cholera immunization. V. Cross-reactive and typespecific determinants on *V. cholerae* Inaba and Ogawa endotoxin and their significance for protective immunity. Acta path. microbiol. scand. Sect. C, *83:* 221–230, 1975.

Immunodiffusion studies demonstrated 7–8 precipitinogenic factors in strains of *Vibrio cholerae*, almost all of which were shared by bacteria of the Inaba and the Ogawa serotype. The endotoxin was shown to contain type-specific as well as cross-reactive (group-specific) precipitinogenic determinants. Quantitative inhibition studies by several techniques showed, however, that both homologous and heterologous endotoxin (purified lipopolysaccharide, LPS, with 10–15 per cent nonseparable protein) could give complete antibody inhibition, but for the same degree of inhibition 3- to 43-fold more of the heterologous than of the homologous LPS was needed. By affinity chromatography using columns with LPS coupled to Sepharose beads, it was possible to purify antibodies against *V. cholerae* LPS and to separate the antibodies against the group-specific and the type-specific endotoxin determinants. Studies by the small bowel loop technique in rabbits showed that antisera and purified anti-endotoxin antibodies protected against both homologous and serotype heterologous experimental cholera. Antibodies to the type-specific endotoxin determinant protected only against homologous infection, whereas the purified group-specific antibodies gave increased resistance against infection with both Inaba and Ogawa bacteria. Subcutaneous immunization of rabbits with formalin-killed vibrios induced a significant level of immunity against homologous infection. Some protection against the heterologous serotype was also observed, which however, was of less magnitude than the homologous immunity.

Key words: Cholera immunization; *V. cholerae;* protective immunity; Inaba-Ogawa relationship.

A.-M. Svennerholm, Institute of Medical Microbiology, Guldhedsgatan 10, 41346 Göteborg, Sweden.

Received 10.i.75 Accepted 23.i.75

The cholera vibrios of O group 1 cross-react immunologically through shared group-specific somatic structure(s) (A), but can be differentiated into two major serotypes, Ogawa and Inaba, by the additional presence of type-specific surface determinants (B and C) (2, 20). This distinction into serotypes is operationally useful, although the expression of the type-specific determinants may be quantitative but not qualitative as suggested by cross-absorption experiments (5, 22) and

by observations of reciprocal serological conversions of both serotypes (17, 21). The group-specific and the type-specific antigenic reactivities have both been associated with preparations of endotoxic lipopolysaccharide (LPS) (26), but it has not been established whether they are associated with the same or different molecules.

The contribution of the group-specific and type-specific structures to the immunogenicity of *Vibrio cholerae* endotoxin has not been clarified in detail. In particular the individual roles of these structures in inducing protective immunity to cholera require further attention. In the prospective vaccine field trials undertaken the immunizing agent has usually consisted of a mixture of Ogawa and Inaba vibrios, which has not permitted evaluation of cross-protection against the heterologous serotype. In a few studies monovalent vaccines have been tested, but different information of their cross-protective effect has been reported. Thus, a purified Ogawa LPS preparation used in the 1964 East Pakistan trial, gave rise to some protection against the subsequent Inaba epidemic (1), whereas in a later study in 1968 when both monovalent Inaba and Ogawa vaccines were tested only type-specific protective immunity was demonstrated (15). In a follow-up of the 1968 study, however, two annual injections of Ogawa vaccine was found to give some protection against Inaba infection but only in individuals over the age of five (14). Also from animal experiments contradictory data have been presented concerning the importance of type-specific and cross-reactive immunity for protection. Thus, *Sokhey & Habbu* employing the mouse protection test described a poor effect of monovalent cholera vaccines against serotype heterologous infection (23). In contrast, a high degree of cross-protection was shown by *Yoshioka et al.* using both the mouse protection and the rabbit ileal loop tests (27).

The aim of the present study was to analyse the degree of immunological cross-reactivity between cholera vibrios of the Ogawa and Inaba serotypes as quantifiable in serological systems, and to evaluate the capacity of type-specific and group-specific endotoxin determinants to induce protective immunity against experimental cholera in rabbits.

MATERIAL AND METHODS

Bacterial Strains

The following *V. cholerae* type strains, kindly supplied by Dr. *H. Smith*, The Vibrio Reference Laboratory, Philadelphia, Penn, were used: Ogawa strains 41, 41 B, J6156, 1800 (El Tor), 1824 (El Tor, Rough) and Inaba strains 35A3, J89, 6, J6154, 569 B, 1799 (El Tor), 1843 (El Tor, Rough).

Antigens

Antigen preparations for immunization. Live and formalin-killed bacterial cultures were used as immunizing agents. The bacteria were grown in an antigen-free medium (8) for 18 h at 37° C. The organisms were killed either by adding formalin to 0.5 per cent final concentration and incubating them for 1 h at 37° C, or by boiling the culture for 1 h.

Antigenic preparations for serological analyses. Ultrasonic, freeze-press and veronal buffer extracts as well as heat extract antigens (120° C, 1 h) were essentially prepared as previously described for *E. coli* (10).

Purified lipopolysaccharide (LPS) was prepared from *V. cholerae* strains 35A3, 569B, 41, J6156 and J6154 (18). The series of LPS preparations contained 10–15 per cent protein as measured with the method of *Lowry et al.* (13). Repeated phenol-water extraction and ultracentrifugation did not significantly reduce the protein content.

Antisera

Antisera for serological analyses and affinity chromatography. Antisera against the 35A3, 41, J6156 and J6154 vibrios were obtained by giving adult rabbits 2–3 injections of 10^9 formalin-killed bacteria, followed by 4–6 injections of 10^9 live vibrios. All injections were given intravenously (i.v.) with a 7 day interval. The antisera were obtained by cardiac puncture taken 1 week after the last injection.

Antisera for protection studies. Antisera were produced in adult rabbits by giving 2 i.v. injections of 10^9 formalin-killed Ogawa 41 or Inaba 35A3 bacteria 1 month apart. Sera of bleedings taken 3 weeks after the second injection and heated at 56° C for 30 min were used for the protection studies described below.

Absorption of antisera. For the serological studies absorbed antisera were obtained by adding

rising concentrations (0.25 mg/ml–15 mg/ml) of LPS preparations of the homologous or heterologous serotype. The absorptions were performed at 37° C for 1 h by incubating the heat-inactivated (56° C, 30 min) antiserum with an equal volume of LPS solution in PBS. The supernates obtained after centrifugation were used.

In an analogous manner the antisera produced for protection studies were absorbed with stock suspensions of serotype heterologous bacteria, boiled for 1 h.

Double diffusion-in-gel technique. A microplate modification of the Ouchterlony double diffusion technique was employed (25). Quantitative inhibition of precipitating antibodies was studied using antisera absorbed with LPS as described.

Vibriocidal test. The spot agar plaque technique previously described was used for titration of bactericidal antibodies (12). Quantitative inhibition of antibodies was studied by titrating antisera absorbed with rising concentrations of homologous or heterologous LPS.

Enzyme linked immunosorbent assays (ELISA). Titrations were performed as earlier described employing LPS from the strains 35A3 (Inaba) or 41 (Ogawa) as solid phase antigens (11). Quantitative inhibition with the homologous or heterologous LPS was done with antisera (diluted to five times the ELISA titre), which had been preincubated with the LPS at room temperature for 1 h (11).

Affinity chromatography. Inaba LPS from strain 35A3 was covalently coupled to Sepharose 4B particles over a spacer group, diaminohexane, as described in detail elsewhere (24). The Sepharose-LPS was packed in 10 × 250 mm columns to bed volumes of 8–13 ml and equilibrated with a starting buffer, pH 7.2. Serum samples in 10–20 per cent of the bed volumes were applied to the gel and washed with 3–5 bed volumes of the starting buffer to eliminate unbound material. Specifically bound material was eluted with a pH acetate gradient, pH 7 → 3 (24).

The acid eluates were collected, pooled and together with the fraction of non-bound material neutralized by dialysis against PBS, concentrated against polyethyleneglycol to the original serum sample volume and stored at –20° C until use.

Intestinal Protection Tests

Passive protection by antisera. The protective capacity of antisera against experimental cholera was evaluated by a rabbit small bowel loop technique (4). The animal operation procedure (9) was in the present investigation modified by using twenty-five 5-cm loops in each animal with start approximately 40 cm from the pylorus (19). The 569B Inaba and the 41 Ogawa strains were used for challenge. The bacteria were cultivated in Syncase medium at 37° C for 4–5 h and diluted to 1×10^6 (Inaba) and 2×10^7 (Ogawa) bacteria/ml; in later experiments 4×10^7 Ogawa bacteria/ml were used. These were the lowest cell densities which injected in a volume of 0.5 ml gave maximal fluid accumulation in a loop. In the analyses serial 10-fold antiserum dilutions were mixed with an equal volume of bacterial suspension, and 1.0 ml of the mixture was immediately injected into a loop. Each combination of antiserum dilution and bacteria was tested randomly positioned in 3–6 animals. In each rabbit a loop injected with 1 mg crude cholera toxin (NIH preparation 4493G) and another loop injected with PBS were included as positive and negative controls.

The protective titres were calculated from plots of mean accumulated fluid (ml/cm gut) vs serum dilution, and the dilution giving 50 per cent inhibition of the response was determined (PT_{50}).

Protection by active immunization. For each experiment a litter of 6-week old rabbits was used. It was divided in three groups of 2–3 animals, which received respectively formalin-killed Ogawa bacteria, formalin-killed Inaba bacteria and PBS. Two subcutaneous (s.c.) injections were given with an interval of 2 weeks; the initial dose of bacteria being 2.5×10^8 and the second dose 2.5×10^9 or 5×10^9. The animals were bled prior to immunization and immediately before challenge. One week after the last injection small bowel loops were prepared which were injected with serially 10-fold varied numbers of Inaba (strain 569B) or Ogawa (strain 41) bacteria, cultivated as described above. The mean ED_{50}-values, i.e. the number of bacteria giving a half-maximal fluid response were calculated as described by *Burrows* & *Musteikis* (3). The protective effect of the immunization was determined by comparing the ED_{50}-values of the vaccinated and the PBS-injected control groups.

RESULTS

Antigenic Relationship between Ogawa and Inaba Strains

Immunodiffusion studies. Double diffusion-in-gel analyses of ultrasonic, freeze-press and veronal buffer extracts of the two strains Ogawa 41 and Inaba 35A3 (reference strains) with homologous antibacterial antisera revealed at least 8 separate precipitinogenic factors ($a\text{-}h_{Ogawa}$) in the Ogawa bacteria and 7 such factors ($a\text{-}g_{Inaba}$) in the Inaba bacteria. All of these precipitinogens were demonstrable in the ultrasonic extracts (Fig. 1), whereas 1 or 2 precipitinogenic factors could not be demonstrated in the other two types

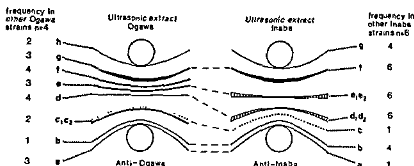


Fig. 1. *Schematic diagrams of double diffusion-in-gel analyses of the antigenic patterns of Inaba (35A3)* and Ogawa (41) bacteria developed with the corresponding antibacterial antisera. Identity reactions between Inaba and Ogawa precipitates in comparative double diffusion are indicated by broken (---) connecting lines. The frequency of the precipitinogenic factors of the reference strains in other Inaba and Ogawa strains are indicated to the left and right in the figure.

of antigen preparations. At least 4 of the Ogawa and 2 of the Inaba antigenic factors were very heat-resistant since they could be demonstrated in bacterial extracts heated to 120° C for 1 h. One of these heat-stable precipitinogens, the factor g_{Ogawa} and the factor f_{Inaba}, identified with the corresponding purified lipopolysaccharide preparations.

Most of the precipitinogenic factors in the 35A3 and 41 bacteria were present also in other Ogawa and Inaba strains, including El Tor variants as demonstrated by comparative immunodiffusion analyses (Fig. 1). Comparative analysis with the ultrasonic extracts of the reference strains as well as with the

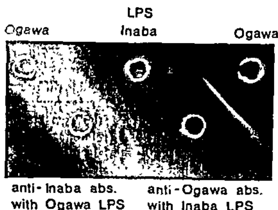


Fig. 2. *Immunodiffusion comparisons of Inaba and* Ogawa LPS by antisera to Inaba and Ogawa bacteria absorbed with LPS of the heterologous serotype.

LPS preparations revealed in all the strains tested except a rough variant of Ogawa (strain 1824), the presence of the g_{Ogawa} or f_{Inaba} precipitinogens.

Comparative double diffusion-in-gel analyses of the ultrasonic extracts of the two reference strains showed that most of the precipitinogenic factors demonstrated were shared by the two serotypes. The lipopolysaccharide precipitinogens of the ultrasonic extracts as well as the purified LPS preparations of the reference strains were serologically indistinguishable on direct comparison (Fig. 1). However, by absorption experiments the presence of additional serotype-specific determinants was demonstrated for the LPS preparations (Fig. 2). By precipitation-in-gel inhibition tests the difference in absorbing capacity of the homologous and the heterologous LPS was quantitated (Fig. 3a). As determined for several antisera against different bacterial strains, a mean of 23 times more Ogawa than Inaba LPS was needed for 50 per cent inhibition (IC_{50}) in the Inaba systems, whereas in the Ogawa systems 43 times more Inaba than Ogawa LPS was required for the inhibition (Table 1).

Vibriocidal tests. Titrations of vibriocidal antibodies gave as mean values for 10 Ogawa antisera 4-fold, and for 12 Inaba antisera 2.5-fold higher titres against homologous than against heterologous bacteria.

Quantitative absorption of vibriocidal an-

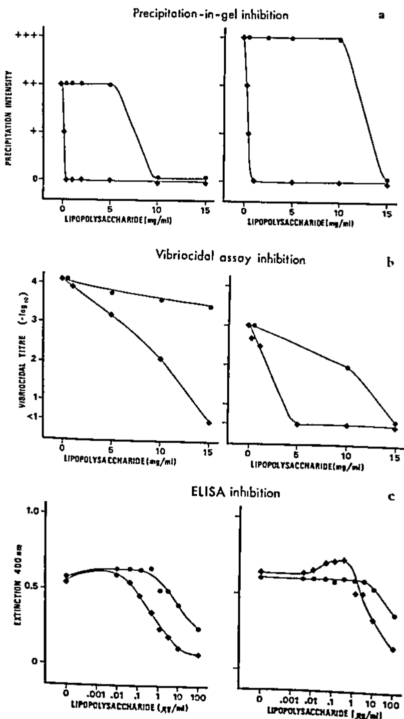


Fig. 3. Quantitative inhibition of anti-Inaba (left) and anti-Ogawa sera (right) with serotype homologous (◆) and heterologous (●) lipopolysaccharide (LPS) preparations. (a) Inhibition of the strength of precipitates formed between the antisera and the homologous LPS. (b) Inhibition of vibriocidal antibody against homologous bacteria. (c) Inhibition of binding of antibodies to homologous solid-phase LPS.

TABLE 1. *Quantitative Inhibition of Anti-Inaba and Anti-Ogawa Sera with Homologous or Heterologous LPS*

Sera	Immunoprecipitation*		Vibriocidal assay§	
	Inaba LPS	Ogawa LPS	Inaba LPS	Ogawa LPS
Anti-Inaba	0.4 ± 0.1†	9.1 ± 1.4	5.3 ± 0.2	>15.5
Anti-Ogawa	12.9 ± 1.5	0.3 ± 0.1	9.7 ± 2.8	2.4 ± 0.1

* Determination of LPS concentration causing 50 per cent inhibition of the strength of precipitates formed between immune sera and serotype homologous LPS (IC_{50}).

§ *Calculation of LPS concentration reducing the homologous vibriocidal titre by 90 per cent* (IC_{90}).

† Mean ± SE, mg/ml, for 2 sera using LPS from 2–3 different strains.

tibodies was done with Inaba and Ogawa LPS (Fig. 3b). As a mean, at least 3 times higher concentration of the heterologous than of the homologous LPS was needed to remove 90 per cent (IC_{90}) of the vibriocidal activity (Table 1).

Enzyme-linked immunosorbent assays. The immunologic cross-reactivity between Ogawa and Inaba LPS was also studied employing the primary binding antibody assay ELISA. Titrations of 10 different anti-Ogawa antisera gave as a mean 2-fold, and of 8 anti-Inaba antisera 6-fold higher titres against the homologous than against the heterologous LPS. Quantitative inhibition studies showed that for antisera against both serotypes, 20–30 times more of the heterologous than of the homologous LPS was needed for 50 per cent inhibition of antibody binding to the solid phase homologous antigen (Fig. 3c).

Affinity chromatography studies. Attempts were made to separate antibodies against the group-specific and the type-specific endotoxin determinants by means of an affinity chromatography column with Inaba LPS coupled covalently to Sepharose. As shown in Table 2 less than 1 per cent of the LPS-reactive antibodies of an anti-Inaba serum passed through the column. Of the antibodies which were bound specifically to the gel up to 90 per cent could be eluted with the pH gradient acetate buffer as determined by ELISA titrations of the eluates (Table 2). These specifically eluted antibodies showed binding also to tubes coated with Ogawa LPS with a titre which was 10 per cent of that registered against Inaba LPS. Application of an anti-Ogawa serum to the Inaba LPS column resulted in direct passage of 35 per cent of the anti-Ogawa antibodies, and less than 1 per cent of these antibodies did react with Inaba LPS in ELISA, indicating that they were almost exclusively directed against the type-specific Ogawa determinant. By the acetate buffer elution, antibodies which were bound to the gel were partially released. These antibodies gave equal titres against Inaba and Ogawa LPS in ELISA indicating that they were directed against the group-specific determinant (Table 2).

Protection against Experimental Cholera

Passive protection by antisera. Since different antibodies had been demonstrated against the group- and type-specific endotoxin determinants, the individual significance of these antibodies for protection against experimental cholera was investigated by the small bowel loop technique. Both anti-Ogawa and anti-

TABLE 2. *Affinity Chromatography of Antisera Using Columns of Sepharose with Covalently Coupled Inaba LPS*

Immune serum	Antibody fraction	
	Passed	Specifically eluted
Anti-Inaba 35A3	1*	85
Anti-Ogawa 41	35	20

* Per cent of homologous antibody titre in whole serum as registered with the ELISA.

TABLE 3. *Protection against Experimental Cholera by Passive Immunization with Antisera*

Serum	Protective titre (PT_{50})*	
	Inaba	Ogawa
Anti-Ogawa	300	300
Anti-Ogawa absorbed with Inaba bacteria	20	40
Anti-Inaba	1,250	125
Anti-Inaba absorbed with Ogawa bacteria	500	<10

* Dilution of antiserum reducing the fluid accumulation in the rabbit small bowel loop assay by 50 per cent in response to challenge with 5×10^5 Inaba 569B or 1×10^7 Ogawa 41 bacteria.

Inaba antisera, which were shown to be devoid of anti-exotoxin antibodies, protected not only against local challenge with homologous bacteria, but also against serotype heterologous infection (Table 3). This indicates that antibodies directed against the group-specific endotoxin determinant(s) are protective, since it has been shown for this type of antisera that the endotoxin-specific antibodies exclusively mediate the protection (24). The degree of protection against homologous and heterologous infection is difficult to evaluate from the performed experiments, since different challenge doses were employed.

Cross-wise absorption experiments showed that also serotype-specific antibodies were protective (Table 3). Thus, absorption of an anti-Inaba serum with heat-killed Ogawa bacteria, eliminating antibodies against the group-specific determinant(s), retained much of the protective activity against Inaba but not against Ogawa infection. An anti-Ogawa serum absorbed with heated Inaba bacteria gave some protection against infection with bacteria of both serotypes, probably due to incomplete absorption.

Passive protection by antibodies separated by means of affinity chromatography. Antibody fractions prepared by affinity chromatography as described above were also tested for protective capacity against Inaba and Ogawa infection. As presented in Table 4,

TABLE 4. *Protection against Experimental Cholera by Antibody Fractions Prepared by Affinity Chromatography*

Antibody fraction	Protective titre (PT_{50})*	
	Ogawa	Inaba
Anti-Inaba§ (anti-A, anti-C)	60	1600
Type-specific anti-Ogawa† (anti-B)	600	0
Group-specific anti-Ogawa$ (anti-A)	25	500

* Dilution of antibody fraction reducing the fluid accumulation in the rabbit small bowel loop assay by 50 per cent in response to challenge with 5×10^5 Inaba 569B or 2×10^7 Ogawa 41 bacteria.

§ Purified anti-Inaba LPS antibody eluted from an Inaba LPS-Sepharose column.

† Anti-Ogawa serum passed through an Inaba LPS-Sepharose column for elimination of cross-reactive (anti-A) antibodies.

$ Purified cross-reactive antibodies from anti-Ogawa serum eluted from the Inaba LPS-Sepharose column as described in c).

the specifically bound and subsequently eluted anti-Inaba LPS antibody fraction, i.e. a mixture of group- and type-specific antibodies (anti-A, anti-C) gave significant protection against both serotypes. Type-specific anti-Ogawa antibodies (anti-B) alone, obtained by running an anti-Ogawa antiserum through the Inaba LPS-column for removal of cross-reactive antibodies, protected with high titre against Ogawa but not at all against Inaba infection. In contrast, the group-specific antibodies (anti-A) from the same serum gave protection against infection by both serotypes (Table 4).

Protection by active immunization. The protective effect of active immunization with formalin-killed Inaba or Ogawa bacteria against homologous and heterologous infection was evaluated in littermate young rabbits (Table 5). Three experiments performed on separate occasions demonstrated that immunization with Inaba bacteria gave 3- to 20-fold higher resistance to homologous challenge than was observed in PBS-injected control animals tested concurrently. In two experiments (no 2 and 3) a 3- to 6-fold higher

TABLE 5. *Homologous and Heterologous Protection in Rabbits by Immunization with Killed Vibrio*

	Immunization	No of animals	Challenge dose for ED_{50}* Inaba $\times 10^{-5}$ bacteria	Ogawa $\times 10^{-7}$ bacteria
	Ogawa§	2	3.2	6.25
Experiment 1	Inaba§	2	12.6	1.25
	PBS	2	4.0	1.25
	Ogawa†	2	0.63	0.32
Experiment 2	Inaba†	2	100	8.0
	PBS	2	5	1.25
	Ogawa†	3	8	0.16
Experiment 3	Inaba†	3	20	0.63
	PBS	3	3.2	0.2

* Number of Inaba 569B or Ogawa 41 bacteria causing half-maximal fluid accumulation in the small bowel loop assay.
§ Two s.c. injections of 2.5×10^9 bacteria given 2 weeks apart.
† Two s.c. injections, the first with 2.5×10^8 bacteria and the second 2 weeks later with 5×10^8 bacteria.

protective effect in immunized than in control animals was also found against heterologous infection (Table 5).

In animals immunized with Ogawa bacteria protection against homologous challenge was only found in one experiment (no 1), whereas in the two other experiments the immunized groups showed a lower resistance than the non-immunized controls. Increased resistance to Inaba bacteria was seen in one experiment (no 3) (Table 5).

DISCUSSION

The immunidiffusion analyses performed on the antigenic composition of a number of strains of *V. cholerae,* showed the presence of 7–? precipitinogenic factors in both Inaba and Ogawa bacteria. Most of these antigens were seemingly identical for the two serotypes and no consistent difference in the antigenic mosaic was noted between classical cholera bacteria and vibrios of the El Tor biotype.

The presence of group-specific (A) as well as of type-specific (C or B) antigenic determinants on *V. cholerae* Inaba and Ogawa endotoxin was demonstrated both directly and by absorption studies using immunoprecipitation, vibriocidal and enzyme-linked immunosorbent techniques. The immunodiffusion studies further indicated that these two types of antigenic specificities reside on the same molecule, since they were associated with a single precipitinogenic factor which could be extracted as lipopolysaccharide (LPS) by the hot phenol-water procedure. The LPS preparation gave despite its content of 10–15 per cent protein, a single precipitate with antisera, which suggests that the protein, if antigenic, is part of the endotoxin molecule. This protein might be identical to the vibriocidal antibody-inducing heat-stable protein antigen described by *Neoh* & *Rowley* (16), since our LPS preparations absorbed all of the vibriocidal antibodies of the anti-*V. cholerae* antisera.

In quantitative absorption studies it was found that 3 to 43 times more of serotype heterologous than of homologous LPS was required for a corresponding degree of inhibition of anti-endotoxin antibodies. With high concentrations of the heterologous antigen it was, somewhat surprisingly, possible to eliminate all of the antibody activity to the homologous endotoxin. These findings are consistent with one of the following explanations: (i) the presence in both Inaba and

Ogawa LPS of low amounts of the type-specific determinant of the heterologous serotype, i.e. AB((C)) for Ogawa and AC((B)) for Inaba, (ii) structural similarities between the type-specific determinants which in antigen excess allow for low binding strength interaction with the heterologous antibodies, or (iii) a low proportion of mutant organisms of the heterologous serotype in the bacterial culture from which the LPS was prepared.

A major purpose of this study was to evaluate the contribution of the group-specific and type-specific determinants for the protective immunogenicity of *V. cholerae* endotoxin. Passive immunization experiments with anti-Inaba and anti-Ogawa antisera, and with such antisera absorbed with serotype heterologous bacteria, indicated that antibodies both against the group- and the type-specific determinants are protective against experimental cholera in rabbits.

To enable protection studies with purified and separated antibodies against the group- and type-specific determinants of *V. cholerae* endotoxin, affinity chromatography of antisera using columns with Sepharose-coupled LPS was undertaken. This approach proved to be highly useful, since such gels specifically bound >99 per cent of the homologous anti-LPS antibodies . Up to 90 per cent of these antibodies could be released by acid buffers. The biological effect of these specifically eluted antibodies seemed to be intact, since their protective capacity was as good as that of the immune serum they were derived from (24). In the present investigation it was found that anti-Inaba LPS antibodies purified from Inaba LPS-gel protected against both homologous and heterologous cholera infection, whereas the type-specific anti-Ogawa antibodies gave only homologous protection. The group-specific antibodies obtained from the anti-Ogawa serum protected against both serotypes. These data support the concept that both type-specific and group-specific antibodies contribute to immunity against cholera.

It has been suggested that locally formed antibodies are of greater importance than serum antibodies for protection (6, 7). This together with the strictly gastro-intestinal nature of the cholera disease initiated experiments to investigate if both the type- and group-specific antibodies do operate functionally in the gut also after active immunization with a killed bacterial vaccine. It was found that immunization of young rabbits with Inaba bacteria consistently gave rise to significant protection against homologous infection. In experiments 2 and 3 when considerable protection against the homologous strain was registered also some protection against Ogawa infection was found (Table 5). In the animals immunized with Ogawa bacteria the results were less clear-cut. Homologous protection was only induced in one of three experiments, whereas in the other two the immunized animals showed even less resistance than non-immunized controls. It is possible that this enhanced susceptibility in the vaccinated animals may have resulted from induction of immunological tolerance, especially as very young animals were employed. However, the serum vibriocidal antibody titres were no lower in the infection-sensitive animals (experiments 2 and 3) than in the protected ones (experiment 1) indicating that if tolerance had been induced, it was restricted to the gut.

The excellent technical assistance of Mrs. *U. Westerberg-Berndtsson* is gratefully acknowledged. We also thank Dr. *L. Myerowitz* and Dr. *J. B. Robbins* for valuable advice concerning coupling of polysaccharides to agarose beads.

This study was supported by grants from the *Swedish Medical Research Council* (16X-3383), the *Faculty of Medicine, University of Göteborg* and the *Ellen, Walter and Lennart Hesselman's Foundation.*

REFERENCES

1. *Benenson, A. S., Mosley, W. H., Fahimuddin, M. & Oseasohn, R. O.:* Cholera vaccine field trials in East Pakistan. 2. Effectiveness in the field. Bull WHO *38:* 359–372, 1968.
2. *Burrows, W., Mether, A. N., McGann, V. G. & Wagner, S. M.:* Studies on immunity to Asiatic cholera. II. The O and H antigenic structure of the cholera and related vibrios. J. Infect. Dis. *79:* 168–196, 1946.

3. *Burrows, W. & Musteikis, G. M.:* Cholera infection and toxin in the rabbit ileal loop. J. Infect. Dis. *116:* 183–190, 1966.
4. *De, S. N. & Chatterje, D. N.:* An experimental study of the mechanism of action of *Vibrio cholerae* on the intestinal mucous membrane. J. Pathol. Bacteriol. *46:* 559–562, 1953.
5. *Finkelstein, R.:* Cholera. Critical Reviews in Microbiology. *2:* 553–623, 1973.
6. *Freter, R.:* Comparison of immune mechanisms in various experimental models of cholera. Bull. WHO *31:* 825–834, 1964.
7. *Freter, R. & Gangarosa, E. J.:* Oral immunization and production of coproantibody in human volunteers. J. Immunol. *91:* 724–729, 1963.
8 *Holm, S. E. & Falsen, E.:* An antigen-free medium for cultivation of β-hemolytic streptococci. Acta pathol. microbiol. scand. *69:* 264–276, 1967.
9. *Holmgren, J., Andersson, A., Wallerström, G. & Ouchterlony, Ö.:* Experimental studies on cholera immunization. II. Evidence for protective antitoxic immunity mediated by serum antibodies as well as local antibodies. Infect. Immun. *5:* 662–667, 1972.
10. *Holmgren, J., Eggertsen, G., Hanson, L. A. & Lincoln, K.:* Immunodiffusion studies on *Escherichia coli.* I. Identification of O, K and H antigens in an O6 strain. Acta path. microbiol. scand. *76:* 304–318, 1969.
11. *Holmgren, J. & Svennerholm, A.-M.:* Enzyme-linked immunosorbent assays for cholera serology. Infect. Immun. *7:* 759–763, 1973.
12. *Holmgren, J., Svennerholm, A.-M. & Ouchterlony, Ö.:* Quantitation of vibriocidal antibodies using agar plaque techniques. Acta path. microbiol. scand. Sect. B, *79:* 708–714, 1971.
13. *Lowry, O. H., Rosebrough, N. Y., Farr, A. L. & Randall, R. Y.:* Protein measurements with the Folin phenol reagent. J. Biol. Chem. *193:* 265–275, 1966.
14 *Mosley, W H, Aziz, K. M. A., Rahman, A. S. M M., Chowdhury, A. K. M. A & Ahmed, A* Field trials of monovalent Ogawa and Inaba cholera vaccines in rural Bangladesh —three years of observation. Bull. WHO *49:* 381 387, 1973
15. *Mosley, W H, Woodward, W. E, Aziz, K. M. A, Rahman, A S M. M., Chowdhury, A K. M. A., Ahmed, A. & Feeley, J. C.:* The 1968–1969 cholera-vaccine field trial in rural East Pakistan. Effectiveness of monovalent Ogawa and Inaba vaccines and a purified Inaba antigen, with comparative results of serological and animal protection tests. J. Infect. Dis. *121* Suppl: S1-S9, 1970.
16. *Neoh, S. H. & Rowley, D.:* The antigens of *Vibrio cholerae* involved in the vibriocidal action of antibody and complement. J. Infect. Dis. *121:* 505–513, 1970.
17. *Nobechi, K., Nakano, E. & Nagao, M.:* Studies on shifting of the serotypes of cholera vibrios. The first report: Studies *in vitro*, in Symposium on Cholera Sponsored by the U.S. Japan Cooperative Medical Science Program Office of International Research, National Institutes of Health, Palo Alto, California July 26–28, 1967, Office of International Research, National Institutes of Health, Bethesda, Maryland 1967, pp. 119–121.
18. *Ørskov, F., Ørskov, I., Jann, K., Müller-Seitz E. & Westphal, O.:* Immunochemistry of *Escherichia coli* O antigens. Acta path. microbiol. scand. *71:* 339–358, 1967.
19. *Pierce, N. F. & Wallace, C. K :* Stimulation of jejunal secretion by a crude *Escherichia coli* enterotoxin. Gastroenterology *63:* 439–448, 1972.
20. *Pollitzer, R.:* Cholera. WHO Monograph Ser. No 43, 1959.
21. *Sack, R. B. & Miller, C. E.:* Progressive changes of vibrio serotypes in germ-free mice infected with *Vibrio cholerae.* J. Bacteriol *99:* 688–695, 1969.
22. *Sakazaki, R. & Tamura, K.:* Somatic antigen variation in *Vibrio cholerae.* Jap J. Med. Sci. Biol. *24:* 93–100, 1971.
23. *Sokhey, S. S. & Habbu, M K.:* Biological assay of cholera vaccine. Bull. WHO *3:* 43–53, 1950.
24. *Svennerholm, A.-M.:* Experimental studies on cholera immunization. IV. The antibody response to formalinized bacteria and purified endotoxin with special reference to protective capacity. Int. Arch. Allergy (in press) 1975.
25. *Wadsworth, C.:* A slide microtechnique for the analysis of immune precipitates in gel. Int. Arch. Allergy *10.* 355–360, 1957.
26. *Watanabe, Y., Verwey, W. F., Guckian, J. C., Williams, H. R., Phillips, P. E & Rocha, S. S.:* Some of the properties of mouse protective antigens derived from *V. cholerae.* Tex. Rep. *27,* Suppl. 1: 275–298, 1969.
27. *Yoshioka, M., Takahashi, K., Hirono, T., Kazuno, Y., Takeuchi, S., Akiba, Y., Ghoda, A., Suzuki, T. & Suzuki, Y.:* Separation of protective bacterial substances against experimental vibrio infection. J. Infect. Dis. *121* Suppl : S45–S49, 1970.

Acta path. microbiol. scand. Sect. C, *83:* 231–237, 1975

DETECTION OF HUMAN ANTIBODIES TO *TRICHINELLA SPIRALIS* BY ENZYME-LINKED IMMUNOSORBENT ASSAY, ELISA

E. Engvall and I. Ljungström

Department of Immunology, Wenner-Gren Institute, University of Stockholm, Norrtullsgatan 16, S-113 45 Stockholm and Department of Parasitology, National Bacteriological Laboratory, S-105 21 Stockholm, Sweden

Engvall, E. & Ljungström, I. Detection of human antibodies to *Trichinella spiralis* by enzyme-linked immunosorbent assay, ELISA. Acta path. microbiol. scand. Sect. C, *83:* 231–237, 1975.

Enzyme-linked immunosorbent assay, ELISA, was used to detect and quantitate antibodies to *Trichinella spiralis* in human sera. An extract of *Trichinella spiralis* larvae was adsorbed to polystyrene tubes. These were incubated with serum and bound antibodies were detected by anti-immunoglobulin labelled with alkaline phosphatase. Class specific conjugates were used to detect specific IgM and IgA antibodies ELISA was at least as sensitive as passive haemagglutination and more sensitive than indirect immunofluorescence.

Key words: *Trichinella spiralis;* antibodies; enzyme-linked immunosorbent assay.

E. Engvall, Dept. of Immunology, Wenner-Gren Institute, University of Stockholm, Norrtullsgatan 16, S-113 45 Stockholm, Sweden.

Received 16.xii.74   Accepted 16.xii.74

In order to establish in man the serodiagnosis of *Trichinella spiralis* infection as well as of certain other parasitic diseases, agglutination tests and/or indirect immunofluorescence are commonly used. Agglutination tests are highly sensitive, but preferentially detect antibodies of the IgM class (7). Indirect immunofluorescence (IFL) is often less sensitive but can be used to detect antibodies of different classes by means of class specific anti immunoglobulin conjugates. In both agglutination tests and IFL, the sera have to be titrated in order to determine the antibody content and the end point of the reaction is a subjective decision.

Enzyme-linked immunosorbent assay, ELISA (4, 5, 6), is a very sensitive and yet simple test. It is objective and quantitative and the result obtained on a single serum dilution gives a relative measure of its antibody content. ELISA has been used for quantitation of antibodies to haptens and to purified antigens (3, 6, 8) and it allows detection of ng quantities of antibodies (6). In the present investigation we have exploited the possibility of using ELISA for the detection of antibodies to a crude antigen prepared by extraction of *Trichinella spiralis* muscle larvae.

Trichinosis is not a common disease in man. However, in 1969, two small outbreaks

of Trichinosis occurred in Sweden, altogether including 25 clinical cases. This provided the opportunity to follow patients clinically (10) and to determine their antibody response by passive haemagglutination (PA) and IFL from a short time after onset of clinical illness (9).

In the present investigation we have tested sera from three of these patients by ELISA. In the sera collected at various times after infection, antibodies of different antibody classes were determined and the results wer compared with those obtained earlier by PA and IFL (9).

MATERIALS AND METHODS

Antigens. Trichinella spiralis larvae were obtained from infected white Sprague-Dawley rats. Infected rat muscle was digested in a solution of 1 per cent pepsin (Difco 1:3000) in 0.06M HCl. The free, washed larvae were used for preparation of soluble antigens according to *Bozicevich et. al.* (2). The larvae were homogenized in saline and extracted for 24 hours at 4° C. The homogenate was centrifuged at $3000 \times g$ for 30 minutes and at $25{,}000 \times g$ for 15 minutes. The protein content was determined by measuring the absorbance at 280 nm, assuming a value of 1.0 for 1 mg/ml protein.

Diaphragms from *Trichinella* infected rats, heavily infected 6–8 weeks earlier, were used as antigen in the indirect immunofluorescence test as described earlier (9).

Sera. Sera from patients with proven *Trichinella spiralis* infection were collected at various times after onset of clinical illness. Sera from persons infected with other nematodes such as *Toxocara, Ascaris* and species of filariae were also tested and the same applies to 30 sera taken at random from a statistically randomized normal sample consisting of sera from 467 healthy Swedes.

Antibodies to human immunoglobulins. Polyspecific rabbit antiserum to human immunoglobulin was obtained by immunizing young adult rabbits twice, 1 month apart, with 0.5 mg $F(ab')_2$ fragments of IgG, emulsified in Freund's complete adjuvant. The $F(ab')_2$ fragments were purified from Gamma-Venin (Behringwerke AG, Mahrburg, W. Germany) by two gel filtrations on Sephadex G-200. 10 days after the last injection, the animals were bled via the ear vein 3–4 times, 3 days apart. The rabbit antibodies were isolated by absorption to insolubilized human IgG and eluted with acid buffer according to *Avrameas & Ternynck* (1).

Rabbit antiserum to IgM was obtained afte immunization with purified IgM[1] as above. Th antiserum was absorbed with insolubilized huma IgG and the antibodies *were finally isolated* by means of an immuno-adsorbent. The latter wa prepared by insolubilizing the fraction of normal human serum excluded by Sephadex G-200.

Rabbit antiserum to IgA was obtained after *immunization with purified human serum* IgA[1]. The antiserum was absorbed with insolubilized IgG and with serum from an IgA deficient individual. The specific IgA antibodies were isolated by means of an immuno-adsorbent consisting of IgA purified from colostrum.

The specificity of the isolated antibodies was checked by immunodiffusion in agar.

Conjugates. Purified rabbit antibodies were conjugated to alkaline phosphatase (Sigma type VII, Sigma Chem. Co., St. Louis, Mo.) by means of glutaraldehyde as described (6).

Enzyme-linked immunosorbent assay, ELISA. ELISA was performed essentially as described in (6). Polystyrene tubes (11×55 mm, Heger Plastics AB, Stallarholmen, Sweden) were coated with *Trichinella spiralis* antigens by incubating each tube with 1 ml of the antigen solution containing 10 µg protein per ml 0.05M carbonate buffer, pH 9.6, either 3 hours at 37° C or overnight at room temperature. Before assay, the tubes were washed twice with saline containing 0.05 per cent Tween 20.

The tubes were then incubated at room temperature with 1 ml serum diluted in phosphate buffered saline pH 7.2 (PBS) with 0.05 per cent Tween 20 and 0.02 per cent NaN_3 for 4–6 hours. After washing the tubes 3 times with saline-Tween they were incubated overnight with a dilution of enzyme labeled anti-immunoglobulin diluted in PBS-Tween. The next morning, the tubes were washed again and the enzyme activity bound to the tubes was measured by addition of 1 ml of the substrate, p-nitrophenyl phosphate (Sigma), 1 mg/ml 1 M diethanolamine buffer at pH 9.8 with 0.5 mM MgCl. After a suitable time, the enzyme activity was stopped by addition of 0.1 ml 2 M NaOH and the absorbance at 400 nm was measured.

In the assay, the conjugates were diluted according to their immune reactivity. Tubes coated with 0.1 µg of immunoglobulin were incubated with conjugate overnight, washed and bound enzyme activity was determined. The polyspecific conjugate and the anti-IgA conjugate were diluted so as to give an absorbance at 400 nm of 0.02 per minute when tested in tubes coated with IgG and IgA, respectively. The anti-IgM conjugate was

[1] The purified preparations of IgM and IgA were kindly supplied by way of a gift from *Dr. K. Heide*, Behringwerke AG).

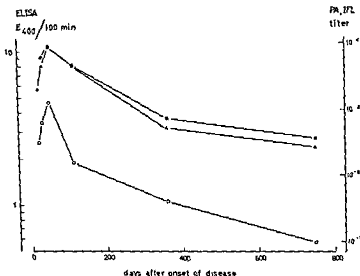


Fig. 1. Antibody response after *Trichinella spiralis* infection in one patient (GY); measured by ELISA (●), PA (▲), and IFL (○).

diluted to give the absorbance of 0.01 per minute in tubes coated with IgM.

Passive haemagglutination (PA) and indirect immunofluorescence (IFL) were performed as described previously (9).

RESULTS

If not otherwise indicated, all sera were tested at dilution 1:400. One normal serum (EE) was included in all experiments as a negative control.

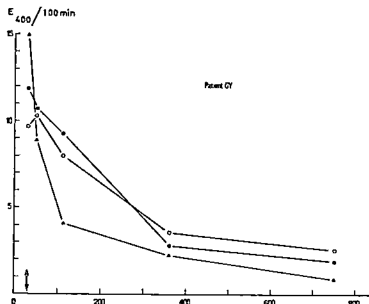
When the 30 randomly chosen normal sera were tested in ELISA it was found that the individual responses obtained did not in any case exceed twice the response obtained with serum EE, which was therefore chosen as a suitable value to discriminate between positive and negative sera. Thus, when the polyspecific conjugate was used, all responses below $E_{400}/100$ min = 1.0 were considered negative. The corresponding values for the IgM specific and the IgA specific conjugates were 1.2 and 0.4, respectively.

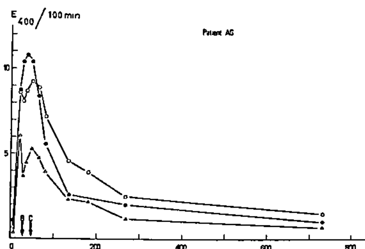
Sera from one patient (GY) taken at various intervals after onset of the disease were tested for antibodies to *Trichinella spiralis* antigen, using the polyspecific anti-immunoglobulin conjugate. The results are shown in Fig. 1 together with the PA and IFL titres of the same sera determined as described in (9). ELISA as well as PA and IFL detect significant amounts of antibodies in the serum of this patient 2 years after infection.

Series of sera from three patients with *Trichinella spiralis* infection were tested by ELISA for specific antibodies of IgM and IgA classes, respectively, by means of class specific conjugates and for total antibody content by means of the polyspecific conjugate. The results obtained are shown in Figs. 2a-c. In two of the patients, AG and RK, from whom early sera were available for testing, antibody production was evident 1–2 weeks after onset of clinical illness. In all three patients, the peak of the immune response was reached within 2 months and antibodies could be detected more than 2 years after infection. These results are in agreement with those obtained before when PA was used (9).

In patients GY and AG, both IgM and IgA antibodies were shown by ELISA to be present 2 years after infection. Tested by IFL, these antibody classes were detectable for only less than 1 year. In patient RK, only the amount of IgA antibodies, but not that of IgM antibodies, was significant after 2 years. This patient had not been tested in IFL by class specific conjugates.

The extinction values obtained by ELISA





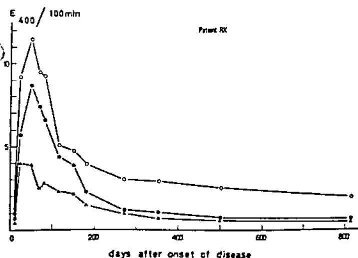
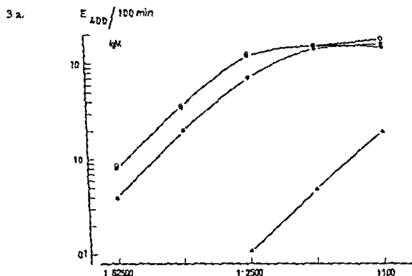


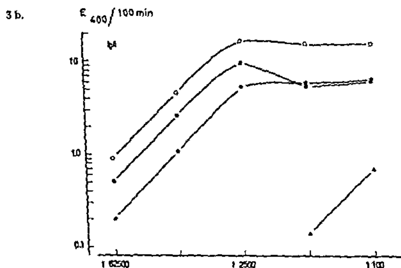
Fig. 2. Antibody response after *Trichinella spiralis* infection in three patients; measured by ELISA by means of conjugate specific for IgM (●), IgA (Δ), and by a polyspecific conjugate (○).

are only related to the amount of serum antibody under conditions of antigen excess. Around the peak of the immune response there may be too much antibody in the serum dilution to fulfil this criterium. The serum samples designated A, B and C in Figs. 2a and b, respectively, were therefore titrated together with the control serum EE. The results are shown in Figs. 3a-c.

As regards low concentrations of antibody there is a straight line relationship between the logarithm of enzyme activity and the logarithm of antibody concentration. In the case of higher concentrations of antibody, the amount of antigen present on the tube becomes a limiting factor and the curves level off.

According to the titrations, serum A from patient GY and serum B from patient AG contained the same amount of specific IgM antibodies while serum C contained less. This agrees with their IgM responses obtained by serum dilution 1:400 (Figs. 2a and b). In contrast, in the case of IgA antibodies,





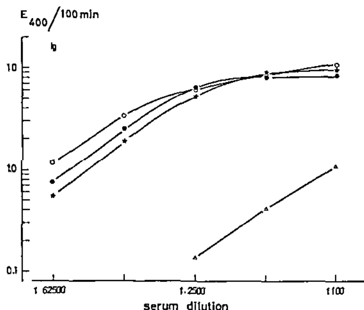


Fig. 3. Titrations of sera in ELISA with IgM specific conjugate (Fig. 3a), *IgA specific conjugate (Fig.* 3b) and with polyspecific conjugate (Fig. 3c). The titrated sera were: ○: serum sample indicated A in Fig. 2a, ●: serum sample B and *: serum C in Fig. 2b. Δ: normal serum (EE).

the titrations show that serum sample B from patient AG contained more antibodies than serum sample C. This is opposite to the results obtained by single serum dilutions.

It is clear from the titration curves (Fig. 3) that sera should be diluted further than 1:400 if more exact data on antibody concentrations are needed during the peak of the immune response.

Sera from persons infected with other nematodes were tested against *Trichinella spiralis* antigen by ELISA, PA and IFL. The results are shown in Table 1. 4/4 of the patients with *Ascaris* infection and 1/1 of those *with Toxocara were negative when tested by* either technique. All five sera from infected individuals with filaria were positive in at least one of the assays (Table 1).

DISCUSSION

We have shown that ELISA can be used for the establishment of the serodiagnosis of *Trichinella spiralis* infection, using a crude antigen as solid phase. ELISA may thus be useful in serodiagnosis of other parasitic infections where little is known about the nature and number of antigens involved. Preliminary work with ELISA with a view to the establishment of malaria, echinococcosis and schistosomiasis has given promising results.

ELISA is useful for screening purposes since one properly chosen serum dilution gives *information about manifest or earlier* infections. At the follow-up of the individual cases, more exact antibody concentrations are obtained by titrations.

ELISA can be used to detect antibodies of single antibody classes. In this respect

TABLE 1. *Antibodies Reacting with* Trichinella spiralis *in Sera from Individuals Infected with Other Nematodes, Tested by ELISA, PA, and IFL*

	Toxocara	Ascaris				Species of filariae				
ELISA	—	—	—	—	—	+	+	+	+	—
PA	—	—	—	—	—	+	+	+	—	+
IFL	—	—	—	—	—	+	+	—	+	—

it would be of special interest to study the IgE response in parasitic infections. The IgE response to *Trichinella spiralis* antigens was not studied in the present work, but has been studied before by radio-allergosorbent test, RAST. Neither a specific nor a nonspecific IgE response was observed (9).

In studies of the class specific immune response, the individual responses of different classes cannot be directly compared. Class specific antibody must be determined in the presence of antigen excess in order to avoid competition between antibodies of different affinities and/or avidities. The apparent decrease in the amount of IgA antibodies at a time when the amount of IgM antibodies is at a maximum, as seen in Fig. 2b, is an artifact due to excess antibody. Because of multivalency, IgM antibodies have a greater chance of reacting with the limited amount of antigen in the tube.

The specificity of ELISA was comparable to that of PA and IFL in that some cross reaction with filaria antigen was seen. The presence of antigens common to the species of filaria and *Trichinella spiralis* could be expected. With a view to removing these, purification of the *Trichinella spiralis* antigen would be desirable.

In the present investigation, the sensitivity of ELISA was comparable with that of PA and higher than that of IFL. It should be mentioned, however, that the IFL results were obtained by conjugates prepared from the immunoglobulin fraction of an antiserum, while ELISA was performed with enzyme labelled purified antibodies. In ELISA, conjugates prepared from purified antibodies are only slightly more effective than those prepared from the whole immunoglobulin fraction (*E. Engvall*, unpublished). Nevertheless, the use of purified antibodies is recommended because this warrants specific reactions of the conjugates.

Some variation of enzyme activities obtained by the same sera in different experiments may be obtained. This variation is easily corrected for by including a standard serum in each experiment.

We are grateful to Mrs. *Sari Feld* for excellent technical assistance.

This work was supported by grant no. 2032-039 *from the Swedish Natural Sciences Research Council.*

REFERENCES

1. *Avrameas, S.* & *Ternynck, T.*: The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. Immunochemistry *6:* 53–66, 1969.
2. *Bozicevich, J., Tobie, J. E., Tomas, E. L., Hoyem, H. M.* & *Ward, S. B.*: A rapid flocculation test for the diagnosis of trichinosis. Publ. Health Reports *66:* 806–814, 1951.
3. *Carlsson, H. E., Lindberg, A. A.* & *Hammarström, S.*: Titration of antibodies to Salmonella O antigens by enzyme-linked immunosorbent assay. Infect. Immun. *6:* 703–708, 1972.
4. *Engvall, E.* & *Perlmann, P.*: Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry *8:* 871–874, 1971.
5. *Engvall, E., Jonsson, K.* & *Perlmann, P.*: Enzyme-linked immunosorbent assay. II. Quantitative assay of protein antigen, immunoglobulin G, by means of enzyme-labelled antigen and antibody-coated tubes. Biochim. Biophys. Acta *251:* 427–434, 1971.
6. *Engvall, E.* & *Perlmann, P.*: Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. J. Immunol. *109:* 129–135, 1972.
7. *Greenbury, C. L., Moore, D. H.* & *Nunn, L. A. C.*: Reaction of 7S and 19S components of immune rabbit antisera with human group A and AB red cells. Immunology *8:* 421–433, 1963.
8. *Holmgren, J.* & *Svennerholm, A.-M.*: Enzyme-linked immunosorbent assays for Cholera serology. Infect. Immun. *7:* 759–763, 1973.
9. *Ljungström, I.*: Antibody response to *Trichinella spiralis*. In Kim C. W. (Ed.): Trichinellosis. Proc. 3rd Int. Conf. on Trichinellosis. Miami 1972. Intext, New York 1974.
10. *Odelram, H.*: A trichinosis epidemic. Scand. J. Infect. Dis. *5:* 293–298, 1973.

Acta path. microbiol. scand. Sect. C, *83:* 238–240, 1975

BRIEF REPORTS

INFLUENCE OF METHYLPREDNISOLONE AND AZATHIOPRINE ON ANTIBACTERIAL SERUM ACTIVITIES AND LEUCOCYTES IN CONVENTIONAL RATS

A. Baardsen

Kaptein Wilhelmsen og Frues Bakteriologiske Institutt, University Hospital, Rikshospitalet, Oslo, Norway

Baardsen, A. Influence of methylprednisolone and azathioprine on antibacterial serum activities and leucocytes in conventional rats. Acta path. microbiol. scand. Sect. C, *83:* 238–240, 1975.

Conventional rats were treated parenterally with high doses of methylprednisolone and/or azathioprine for 21 days. The influence on bactericidal and opsonic activities of serum against ^{32}P-labelled *E. coli* as well as on the number of circulating PMN and lymphocytes was evaluated. Methylprednisolone induced neutrophilia, lymphocytopenia, and reduced the bactericidal activity of serum in one of two strains of rats investigated. Azathioprine induced neutropenia, but did not alter the serum activities studied, except when the dose approximated LD_{50}. The combination of drugs neither altered the blood counts nor the serum activities studied.

Key words: Methylprednisolone; azathioprine; opsonins; leucocytes.

A. Baardsen, Kaptein Wilhelmsen og Frues Bakteriologiske Institutt, University Hospital, Rikshospitalet, Oslo, Norway.

Received 11.ii.75 Accepted 11.iii.75

The infection-enhancing effect of glucocorticosteroids and azathioprine (AZA) might be related to impairment of cellular or humoral factors. The most important functions of the latter are the opsonic and bactericidal activities (1). In previous investigations, the influence of methylprednisolone (MP) and AZA on antibacterial serum factors (1, 2) and circulating leucocytes (4) has been studied on rats living in a controlled environment, i.e. germfree and monocontaminated rats. In conventional individuals, however, the consumption of antibacterial factors may vary with the uncontrolled microbiological environment. In the clinical situation, these drugs may also be administered in combination. Information about the influence of the drug combination is therefore warranted.

The aim of the present *in vivo* study on conventional rats was to evaluate the influence of MP and/or AZA on the opsonic and bactericidal activity of sera against ^{32}P-labelled *E. coli*. In addition, the influence on circulating PMN and lymphocytes was evaluated.

Materials and Methods

Conventional rats, 3-4 months of age, of two strains, CDF (4) and Wistar, were divided into groups as listed in Table 1: a) rats treated with MP 40 mg per kg per day (MP40), b) rats treated with AZA 40 mg per kg per day (AZA40), c) rats treated with AZA 60 mg per kg per day (AZA60), d) rats treated with MP10 + AZA20, f) non-treated controls.

All rats were treated for 21 days by one daily injection of either drug or a combination of drugs. MP was injected s.c. (4) and AZA was injected i.p. (4).

Weights, leucocyte counts, and differential counts of blood leucocytes were recorded on day 0 and day 21 (4). Blood was collected by exsanguination (1) 24 hours after the last medication.

Sera from rats within the individual groups were pooled and stored at –20° C.

For the study of opsonic activity of serum, peritoneal PMN were obtained from non-treated CDF rats (8). The methods for determination of opsonic and bactericidal activity of serum against *E. coli* (8), and treatment of serum by mercaptoethanol (1, 2) have been previously described. The two-samples ranks test was used for statistical analysis (5).

Results and Discussion

The rates of mortality and weight figures are shown in Table 1. In all treated groups, the rats had ruffled fur and showed signs of "failure to thrive".

Haematological data are presented in Table 2. In controls, and in treated rats on day 0, PMN varied from 1200 to 2600, while the corresponding mean of lymphocytes varied from 3600 to 8700. This considerable variation might be related to the presence of microorganisms in an uncontrolled environment (4). MP induced a significant ($p<0.01$) increase in the number of circulating PMN and a significant ($p<0.01$) reduction in the number of circulating lymphocytes (based on comparison of counts on day 0 and day 21). AZA significantly ($p<0.01$) reduced the number of PMN. These haematological observations have been discussed in a previous communication including germfree and monocontaminated rats (4). The present study revealed that the drug combination MP40 + AZA20 did not induce any significant alteration of either PMN counts ($p>0.10$) or lymphocyte counts ($p>0.10$). This might be explained by opposite effects of MP and AZA on the number of circulating PMN. The lympho-

TABLE 1. *Protocol for Animal Experiments*

Strain	Group*	No. of rats	Dead rats	Weight gain per cent
Wistar	MP40	10	0	–7
	AZA40	10	0	+3
	Controls	10	0	+29
CDF	MP40	13	0	–12
	AZA40	14	1	–2
	AZA60	6	2	–24
	MP40 + AZA40	5	5	
	MP40 + AZA20	6	0	–19
	Controls	15	0	+31

* For details see Materials and Methods.

TABLE 2. *Total White Blood Cell Counts (WBC) and Absolute Number of PMN and Lymphocytes per mm³ Blood from Conventional CDF Rats. Influence of Treatment with MP and/or AZA*

Treatment*	Day§	WBC ± SEM/n	PMN ± SEM/n	Lymphocytes ± SEM/n
Controls	0	9836 ± 801/14	1525 ± 222/14	8067 ± 662/14
Controls	21	7707 ± 554/14	2616 ± 350/14	5664 ± 369/15
MP40	0	9414 ± 840/14	1486 ± 173/13	7363 ± 709/13
MP40	21	7123 ± 429/13	3586 ± 235/13	3266 ± 306/13
AZA40	0	10400 ± 944/14	1348 ± 193/14	8690 ± 900/14
AZA40	21	5817 ± 462/12	571 ± 120/12	5151 ± 400/12
AZA60	0	9183 ± 455/ 6	1548 ± 54/ 6	7398 ± 395/ 6
AZA60	21	3733 ± 763/ 3	172 ± 66/ 3	3489 ± 735/ 3
MP40 + AZA20	0	5450 ± 454/ 6	1233 ± 177/ 6	3671 ± 202/ 6
MP40 + AZA20	21	5360 ± 393/ 5	1249 ± 271/ 5	4024 ± 441/ 5

* For details see Materials and Methods

§ Day of treatment or observation period

SEM = standard error of the mean, n = number of observations.

TABLE 3. *Bactericidal and Opsonic Activity of Sera from Control Rats and Rats Treated with MP and/or AZA*

Strain	Group*	Bactericidal activity per cent ± 1SD (n)	Opsonic activity per cent ± 1SD (n)
	MP40	98.8 ± 6.1 (11)	102.6 ± 21.9 (8)
Wistar	AZA40	106.3 ± 4.0 (12)	105.2 ± 20.6 (8)
	Controls	100 ± 4.3 (12)	100 ± 15.3 (8)
	MP40	78.8 ± 10.2 (24)	86.0 ± 8.0 (4)
	AZA40	94.5 ± 21.3 (23)	112.2 ± 30.5 (5)
CDF	AZA60	73.2 ± 4.1 (6)	N.D.
	MP40 + AZA20	102.7 ± 15.0 (6)	126.5 ± 30.6 (7)
	Controls	100 ± 11.0 (26)	100 ± 17.6 (11)

* Fordetails see Materials and Methods.
(n) = Number of observations.
SD = Standard deviation.
N.D. = Not done.

cyte counts in the group of rats treated with MP40 + AZA20 are difficult to explain, and further studies of germfree rats seem to be necessary.

The results on serum activities (Table 3) refer to a final dilution 1:20 for opsonic activity, and 1:10 for bactericidal activity of pooled serum. (Only traces of bactericidal activity were found at 1:100 of control serum, and no bactericidal activity at 1:1000). In control serum treated with mercaptoethanol (1) there was no measurable antibody activity (IgG) at 1:10 when serum from germfree rats was added as a source of complement (1).

Treatment with either drug did not impair the overall serum activities against *E. coli* in Wistar rats. In CDF rats, MP40 significantly ($p<0.01$) reduced the bactericidal activity to 79 per cent. The opsonic activity of the same sera was also slightly, but insignificantly ($p>0.10$), reduced AZA60 reduced the bactericidal activity to 73 per cent, however, this dose approximated LD50 (Table 1). AZA 40 neither affected the bactericidal nor the opsonic activity ($p>0.10$).

The MP-induced reduction of bactericidal activity was consistently found in 3 series of CDF rats. The serum activities tested are mainly mediated by antibodies and complement, while the possible role played by nonspecific humoral factors (7) apart from complement in this test system, is shown to be negligible (1, 2). In a previous study on germfree rats, administration of MP40 simultaneously and after monocontamination with *E. coli* did not impair the activity/production of specific antibodies (2). Furthermore, MP40 did not reduce the activity/production of complement in germfree rats (2). Therefore, it seems unlikely that the present reduction of bactericidal activity is due to an impairment of the ongoing production of antibodies in conventional rats harbouring *E. coli*. Nor is it likely that the synthesis of complement is reduced. On the other hand, an MP-induced increase of the catabolic rate or consumption of antibodies and complement appear more likely in wasting (2) rats probably suffering from infection. Different bacterial populations in rats living in an uncontrolled environment might also explain why the MP-induced reduction of bactericidal activity was not consistently found in both strains of rats, and within the same strain, when MP40 was combined with AZA20. A blocking effect of MP on preformed serum factors (3, 6, 7) is unlikely since serum was collected 24 hours after the last medication and therefore is considered to contain negligible amounts of the drug.

References: 1. *Baardsen, A., Midtvedt, T & Trippestad, A.:* Acta path. microbiol. scand. Sect. B, *81:* 799–805, 1973.—2. *Baardsen, A., Midtvedt, T. & Trippestad, A.:* Acta path. microbiol. scand. Sect. B, *83:* 37–44, 1975. In press.—3. *Baardsen, A., Trippestad, A. & Midtvedt, T.:* Acta path. microbiol. scand. Sect. B, *82:* 696–700, 1974.—4. *Baardsen, A., Midtvedt, T. & Trippestad, A :* Acta path. microbiol. Sect. B. In press.—5. *Mainland, D.:* Elementary medical statistics. 2. ed. W. B. Saunders Company, Philadelphia and London, 1964.—6. *Olds, J. W., Reed, W. P., Eberle, B. & Kisch, A. L.:* Infection and Immunity, *9:* 524–529, 1974.—7. *Pruzanski, W. & Saito, S.:* J Natl Cancer Inst, *52:* 643–647, 1974.—8. *Trippestad, A. & Midtvedt, T.:* Acta path. microbiol. Sect. B, *74:* 259–273, 1968.

Acta path. microbiol. scand. Sect. C, *83:* 241–243, 1975

PURIFICATION FROM EUGLOBULIN OF THE FIRST COMPONENT (CI) OF COMPLEMENT BY HEPARIN, SURAMIN AND LIQUOID PRECIPITATION

G. von Zeipel, Hanna-Stina Hanson and L.-V. von Stedingk

The Central Microbiological Laboratory of Stockholm County, Stockholm, Sweden

Zeipel, G. von, Hanson, H.-S. & Stedingk, L.-V. von. Purification from euglobulin of the first component (CI) of complement by heparin, suramin and Liquoid precipitation. Acta path. microbiol. scand. Sect. C, *83:* 241–242, 1975.

Addition to human euglobulin (EU) of either heparin, suramin or sodium polyanetholsulphate (Liquoid) precipitated most of CI at an ionic strength of 0.215 μ. By heparin, CI was precipitated with 18 per cent of the total EU protein and by suramin and Liquoid with 55 and 64 per cent, respectively. The heparin precipitate was solubilized at 0.515 μ and contained CI of a four-fold higher purity than did EU. CI was recovered by successive extractions of the suramin precipitate at 0.515, 0.765 and 1.15 μ. Purification was two to three-fold. Only small amounts of CI were released from the Liquoid precipitate even at 1.15 μ. The CI-heparin extract was reprecipitated by lowering the ionic strength to 0.215 μ, solubilized at 0.515 μ and filtered on DEAE-Sephadex A 25 to remove heparin. The final CI preparation was six-fold purified at a 50 per cent yield with respect to the CI activity of the initial EU preparation.

Key words: First complement component (CI); purification; heparin, suramin, Liquoid precipitation.

G. von Zeipel, Virus Department of the Central Microbiological Laboratory of Stockholm County, P.O. Box 177, S-101 22 Stockholm, Sweden.

Received 7.iv.75 Accepted 15.iv.75

Materials and Methods

Fresh sera pooled from three blood donors were used.

Euglobulin. EU was prepared according to *Tamura & Nelson* (1968) and *Vroon et al.* (1970). 200 ml serum, pH 7.5, was diluted 1:5 with cold 0.005 M phosphate buffer containing 0.15 mM $CaCl_2$. The mixture was stirred at 0° C for 30 min until centrifuged at 15,000 *g* for 30 min. The precipitate was washed twice with the same buffer and dissolved in 10 ml of this buffer containing 0.3 M NaCl.

Heparin (170 USP units/mg) was obtained from Sigma, USA, suramin from Bayer, Germany, Liquoid from Hoffmann-La Roche, Switzerland and DEAE-Sephadex A 25 from Pharmacia, Sweden.

Complement esterase (CIs). One unit of esterase was the amount that liberated 0.5 micromoles of H^+ from 0.02 M N-acetyl-L-tyrosine-ethyl ester in 15 min at pH 7.5 and 37° C as described by *von Zeipel et al.* (1974).

Clq, IgG and IgM were assessed on EDTA-treated samples by single radial immunodiffusion (*Mancini et al.* 1965) in plates containing 1 per cent agarose, 1 to 2 per cent antiserum (Behringwerke, Germany), 10 mM EDTA, 0.05 M Tris-glycine buffer, pH 8, and 0.15 M NaCl.

Protein was measured with the Folin phenol reagent (*Lowry et al.* 1951).

Heparin was determined according to *Yin et al.* (1973) employing reagents of the Sigma kit (Sigma, USA, Technical bulletin No. 870).

Preparative procedures were carried out at +4° C.

TABLE 1. *Precipitation of CI from Euglobulin by Heparin, Suramin and Liquoid. Purification of CI Indicated by Purification of CIs and Clq*

Protein fraction	Protein mg	Tot. No. of CIs units	CIs units/ mg protein	Purification (×)		Recovery in per cent	
				CIs	Clq	CIs	Clq
Euglobulin (at 0.215 μ)	25	1425	57	1	1	100	100
1st heparin ppt.,* sol.§ at 0.515 μ	4.5	1069	237.6	4.2	2.6	75	47
2nd heparin ppt., sol. at 0.515 μ	2.5	889	355.6	6.2	3.4	62.4	34.0
Suramin ppt.:							
1st extr.† at 0.515 μ	4.3	257	59.8	1	–	18	–
2nd extr. at 0.765 μ	3.9	667	171	3	2.8	46.8	43.0
3rd extr. at 1.15 μ	3.0	354	118	2	2.5	24.8	31.0
Liquoid ppt.:							
1st extr. at 0.515 μ	4	44	11	0.2	–	3	–
2nd extr. at 0.765 μ	1.9	55	28.9	0.5	–	3.9	–
3rd extr. at 1.15 μ	1.5	78	52	0.9	–	5.5	–

* ppt. = precipitate.
§ sol. = solubilized.
† extr. = extract.
– = not done.

Buffer solutions contained 0.01 per cent sodium azide.

Results

A single batch of EU was used in the experiment recorded in Table 1. The ionic strength of EU was adjusted from 0.315 μ to 0.215 μ and the solution centrifuged at 1000 *g* for 30 min. The precipitate which contained 21 per cent of the initial CI activity of EU was discarded.

The supernatant was divided into three 10 ml volumes to which either heparin, suramin or Liquoid were added to a final concentration of 0.1 mg per ml. The preparations were stirred for 60 min and centrifuged at 1000 *g* for 30 min. The supernatants were removed and the sediments stirred overnight with 5 ml each of NaCl buffer of an ionic strength of 0.515 μ and then centrifuged. The heparin precipitate was completely dissolved in contrast to the suramin and Liquoid precipitates. These underwent two further extractions with NaCl buffers of ionic strengths 0.765 μ and 1.15 μ, respectively. After the last extraction, the suramin precipitate was almost completely dissolved. About half of the Liquoid precipitate, however, remained undissolved.

The purification of CI was analysed on EDTA treated fractions (results on precipitates are given in Table 1) by determinations of CIs and Clq.

Analyses of CIs of the supernatants (not shown in the Table) showed that heparin, suramin and Liquoid had precipitated 81, 92 and 98 per cent, respectively, of the CI content of EU and 18, 55 and 64 per cent, respectively, of the EU protein.

Most of the CI activity of the heparin precipitate was recovered by extraction at 0.515 μ and found to be four-fold purified. The suramin precipitate released 19, 51 and 27 per cent of its CI content, purified two to three-fold, when successively extracted at 0.515 μ, 0.765 μ and 1.15 μ, respectively. Altogether 13 per cent of the CI was recovered from the Liquoid precipitate in an impure state.

The heparin extract was reprecipitated (2nd precipitate, Table 1) without further addition of heparin, by lowering the ionic strength to 0.215 μ. This resulted in a 50 per cent increase of purity of CI.

The first and second heparin precipitates contained 0.04 and 0.02 mg, respectively, of heparin per mg of protein.

Heparin was removed from the dissolved second precipitate on passage at an ionic strength of 0.515 μ through a DEAE-Sephadex A 25 column 9 × 0.9 cm. The final preparation of CI was contaminated by 1.6 per cent IgG and 2.2 per cent IgM. The yield was about 50 per cent of the CI content of the initial EU preparation (not shown in the Table).

Comments

The procedures outlined above by which to purify CI are based on the well known interaction between CI and polyanions (for references see *Rent et al.* 1975). Among the polyanions tested, heparin apparently was more suitable than suramin and Liquoid for the present purpose in giving a more specific precipitation of CI from EU and ensuring milder conditions for a recovery of an intact CI complex.

References: 1. *Lowry, O. H., Rosebrough, N. J., Farr, A. L. &Randall, R. J.:* J. Biol. Chem. *193:* 265–275, 1951.—2. *Mancini, G., Carbonara, A. O. & Heremans, J. F.:* Immunochemistry *2:* 235–254, 1965.—3. *Rent, R.-M., Ertel, N., Eisenstein, R. & Gewurz, H.:* J. Immun. *114:* 120–124, 1975. —4. *Tamura, N. & Nelson, R. A.:* J. Immun. *101:* 1333–1345, 1968.—5. *Vroon, D. H., Schultz, D. R. & Zarco, R. M.:* Immunochemistry *7:* 43–61, 1970.—6. *Yin, E. T., Wessler, S. & Butler, J. V.:* J. Lab. Clin. Med. *81:* 298–310, 1973.—7. *von Zeipel, G., Hanson, H.-S. & von Stedingk, L.-V.:* Acta path. microbiol, scand. Sect. B, *82:* 590–592, 1974.

Acta path. microbiol. scand. Sect. C, *83:* 243–245, 1975

QUANTITATIVE PRECIPITATION IN MICROTITER PLATES

Bent Aasted

Immunological Laboratory, Blood Grouping Department, Statens Seruminstitut, Copenhagen, Denmark

Aasted, B. Quantitative precipitation in microtiter plates. Acta path. microbiol. scand. Sect. C, *83:* 243–245, 1975.

A quantitative precipitation micro-technique has been developed using the plastic microtiter plates. Amounts as small as 80–160 µl of antiserum were sufficient for the determination of the precipitin curve. The microprocedure was shown to correlate both with the normal quantitative precipitation technique and the single radial immunodiffusion test.

Key words: Quantitative precipitation; microtiter plates.

Bent Aasted, The Rockefeller University, New York, N.Y. 10021, U.S.A.

Received 12.iii.75 Accepted 26.iv.75

The quantitative precipitation technique originally described by *Heidelberger & Kendall* (1929) has been a very useful test for quantitating antibodies, and the test has been adapted to numerous antigen-antibody systems. The technique has the advantage that antibodies can be measured in a well defined unit such as mg antibody per ml antiserum. Precipitation inhibition tests (*Krause & McCarty* 1962) and coprecipitation tests (*Bokisch et al.* 1972) have also been shown to be useful precipitation techniques, but the overall disadvantage of these techniques is the rather large amounts of both antigen and antiserum required.

The aim of the studies presented in this report has been to reduce the amount of reagents required for the analyses by performing the precipitation reaction in the small wells present in the microtiter plates originally designed for microagglutination titration studies. The present report describes the successful use of these plates in microprecipitation studies with antisera against streptococcal group A and C carbohydrate antigens.

Materials and Methods

Antisera: 24 Rabbit antistreptococcal group A antisera and 24 antistreptococcal group C antisera were used throughout the study. Both groups of antisera consisted of 12 antisera obtained after a primary immunization schedule and 12 antisera obtained after a secondary immunization schedule. The preparation of the antisera with heat-killed, pepsin treated streptococci and the immunization schedules have been described before (*Aasted* 1974a).

Antigens: Formamide extracts of group A and C streptococci according to *Fuller* (1938) were used as antigens. The antigen solutions had a rhamnose content of 64 μg/ml solution, as determined by the cysteine-sulfuric acid method (*Dische & Shettles* 1948). The streptococcal extracts were shown to contain some protein which, however, could be separated from the carbohydrate antigen by sephadex G-200 chromatography.

Protein was determined by the Biuret method (*Gornall et al.* 1949).

Single radial immunodiffusion test was carried out as described previously (*Aasted* 1974a).

Quantitative precipitation technique in macroscale was performed in glass tubes as described previously (*Aasted* 1974a).

Quantitative precipitation technique in microscale was performed using micro-titration plates with U wells* instead of the glass tubes normally used in the macroquantitative precipitation test. The micro-procedure followed the same principles as described for the macrotest (*Aasted* 1974a), but only with 10–20 per cent of the amount of antigen and antiserum. Twelve experiments were carried out using one plate with seven 2 fold dilutions of 100 μl samples of antigen and one control well with 100 μl of phosphate buffered saline pH 7.38 (PBS). 20 μl samples of primary antisera and 10 μl samples of secondary antisera were added to the wells using a Hamilton syringe with dispensor. After mixing antigen with antiserum by careful shaking, the plates were incubated at 37° C for 30 minutes and then stored for 2 days at 4° C, with careful shaking after one day. After the incubation period, the plates were centrifuged at 3000 rev/min (r = 15 cm) for 15 minutes and 10 μl samples of the supernatants were placed in micro-Ouchterlony plates (Gelman equipment) for determination of the antigen or antibody excess zones. The remaining supernatants were then sucked away by a Pasteur pipette and the precipitates were washed three times with PBS. After the last washing procedure, the precipitates were dissolved in 150 μl of 0.2 N NaOH and 150 μl of Biuret reagent were added. The absorbance was measured with MT2 cuvettes in a Zeiss spectrophotometer at 540 nm.

* C. A. Greiner und Söhne, Kunstoffwerke, W. Germany.

Results and Conclusions

The total micro-analysis required only 80–160 μl of antiserum, while the normal macro-procedure requires 800 μl. By comparing the amount of precipitins measured by the micro-technique with the amount measured by the normal macro-procedure using the primary and secondary antisera against streptococcal group A and C carbohydrates, highly significant correlation was found between the two procedures. The correlation coefficients between the two tests were calculated to be 0.84 for group A precipitins and 0.82 for group C precipitins (Table 1). The standard deviations of the micro and macro-tests were calculated on the basis of double determinations to be $S.D._{macro}$ = 4.2 per cent (12 determinations) and $S.D._{micro}$ = 13.0 per cent (14 determinations). Thus, it is seen that the micro-technique was not as reproducible as the macro-technique, which was to be expected when the amount of the reagents was reduced to 10–20 per cent.

By obtaining the proportionality lines between precipitins measured by the two techniques it was found, that the group A line fitted well, while the group C line did not. More precipitate was measured by the micro than by the macro-test. It would be obvious to postulate that the washing procedure, which is the most time consuming and difficult step in the micro-procedure, has not been sufficient to wash out all non-specific protein present in the serum. This was shown not to be the case, since 10 washing steps instead of 3 gave the same results. However, the lack of proportionality may be explained by the presence of anti-antibodies in most of the group C antisera (*Aasted* 1974b).

In order to investigate the problem further, a third technique, viz. the single radial immunodiffusion test was included in the analyses. This

TABLE 1. *Comparative Studies between Precipitins Measured by the Micro and Macro-quantitative Precipitation Tests (q.p. tests) and the Single Radial Immunodiffusion Test (s.r.i. Test) Expressed as Correlation Coefficients (r-values)*

	Precipitins	
	Group A	Group C
Micro q.p. test vs macro q.p. test	0.84	0.82
Micro q.p. test vs s.r.i.test	0.87	0.94
Macro q.p. test vs s.r.i. test	0.93	0.86

There were 24 antisera in each group.

test is known to be a quantitative technique for measuring antibodies or antigens (*Mancini et al.* 1965). Besides, the presence of anti-antibodies in antiserum is not to be expected to interfere with the quantitation of the antistreptococcal antibodies in this test, since the anti-antibodies are always present in a smaller amount than the antistreptococcal antibodies (*Bokisch et al.* 1972). They are therefore expected to form a precipitin ring within the ring formed by the antistreptococcal antibodies. The diameter of the outer precipitin ring was therefore used as a measurement of antistreptococcal antibodies. The correlation coefficients between all three tests are presented in Table 1. As can be seen, there was a better correlation between the precipitins measured by the micro and macro-quantitative precipitation technique and those measured by the single radial immunodiffusion test than when they were compared mutually. This will be taken as an indication of the interference of anti-antibodies in the quantitative precipitation in both micro and macro-tests.

Considering that the lack of proportionality found when using the group C antisera may be explained as a peculiarity of the serological system, a micro-technique for quantitating precipitins with a high reproducibility has been developed using no more than 80–160 μl of antiserum as shown for the group A antibody quantitation. The method will be useful as a pilot assay for determining the optimal proportion between antigen and antiserum. Furthermore, it should be convenient in precipitation inhibition and coprecipitation studies, both of which are techniques normally requiring large amounts of antiserum

This study was supported by *Statens Naturvidenskabelige Forskningsraad* (J. No. 511–3569). I wish to thank Dr. *J. V. Spärck* for revising the manuscript, *M. Weis Bentzon* (actuary) for advice on the statistical analyses, and Miss *E. Nielsen* for skilful technical assistance.

References: 1. *Aasted, B.:* Scand. J. Immunol. *3:* 545–552, 1974a.—2. *Aasted, B.:* Scand. J. Immunol. *3:* 553–558, 1974b.—3. *Bokisch, V. A., Bernstein, D. & Krause, R. M.:* J. exp. Med. *136:* 799–815, 1972.—4. *Dische, Z. & Shettles, L. B.:* J. biol. Chem. *175:* 595–603, 1948.—5. *Fuller, A. T.:* Brit. J. exp. Path. *19:* 130–139, 1938.—6. *Gornall, A. G., Bardawill, C. J. & David, M. M.:* J. biol. Chem. *177:* 751–766, 1949.—7. *Heidelberger, M. & Kendall, F. E.:* J. exp. Med. *50:* 809–823, 1929. —8. *Krause, R. M. & McCarty, M.:* J. exp. Med. *115:* 49–62, 1962.—9. *Mancini, G., Carbonara, A. O. & Heremans, J. F.:* Immunochem. *2:* 235–254, 1965.

Acta path. microbiol. scand. Sect. C, 83: 246–248, 1975

HISTOCHEMICAL DEMONSTRATION OF ACID β-GLUCURONIDASE AND CARBOXYLIC ESTER HYDROLASE ACTIVITY IN SENSITIZED LYMPHOCYTES DURING LYSIS OF ALLOGENEIC FIBROBLASTS

Poul Brix Poulsen and Flemming Güttler

Institute of Medical Microbiology, 22 Juliane Maries Vej, DK-2100 Copenhagen Ø, Denmark

Poulsen, P. Brix & Güttler, F. Histochemical demonstration of acid β-glucuronidase and carboxylic ester hydrolase activity in sensitized lymphocytes during lysis of allogeneic fibroblasts. Acta path. microbiol. scand. Sect. C, 83: 246–248, 1975.

Sensitized spleen cells were incubated with ^{51}Cr-labelled target fibroblasts. The interaction of lymphoid cells and target cells was stopped by fixation in formalin vapour. Target cell lysis was evaluated by the release of ^{51}Cr and the cultures were stained for acid β-glucuronidase and carboxylic ester hydrolase. The sensitized lymphoid cells aggregated around cultured donor-type fibroblasts and showed a marked staining for β-glucuronidase activity within a six-hour incubation period. Spleen cells of unimmunized mice were negative or weakly positive for β-glucuronidase. Staining for carboxylic ester hydrolase activity was observed only in spleen cells of allo-immunized mice. The staining was less intense as compared with the staining for β-glucuronidase. These observations add further support to the suggestion that lysosomal hydrolases might have a bearing on cell-mediated target cell injury.

Key words: Lymphoid cells; acid β-glucuronidase staining; carboxylic ester hydrolase staining; allogeneic fibroblasts; lysis.

F. Güttler, Institute of Medical Microbiology, University of Copenhagen, 22 Juliane Maries Vej, DK-2100 Copenhagen Ø, Denmark.

Received 19.iv.75 Accepted 9.v.75

The suggestive role of lysosomal acid hydrolase in cell-mediated cytolysis has been received elsewhere (*Weissmann & Dukor* 1971, *Biberfeld* 1971, *Brondz et al* 1973, *Cohnen et al.* 1973). Recently, we have demonstrated staining for acid phosphatase activity in lymphocytes mediating cytolysis *in vitro* (*Poulsen et al.* 1975). The appearance of staining was immunologically specific and could be correlated to the cytotoxic reaction by the ^{51}Cr release assay (*Cerottini & Brunner* 1971). The histochemical technique employed resulted in a coloured product localized as distinct granules in the perinuclear area. Several acid hydrolases, e.g. acid phosphatase, β-glucuronidase and cathepsin, possess distinct sedimentation characteristics suggesting their existence in a distinct particle, the lysosome (*de Duve* 1969). To document further on the probable activation of lysosomal acid hydrolases during cell-mediated cytolysis, we have studied the staining for acid β-glucuronidase and carboxylic ester hydrolase activity of immune spleen cells interacting with the appropriate target cells.

Materials and Methods

C57BL/6J (H-2^b) mice were immunized by intraperitoneal injection of 10^7 L-cells (CTC clone 929) (H-2^k). The spleens were removed 10 days later and spleen cell suspensions prepared as described previously (*Poulsen et al.* 1975). Control mice were either unimmunized, or mice injected intraperitoneally with 5×10^6 isogeneic EL-4 (H-2^b) tumour cells or 5×10^6 allogeneic DBA-2 P815 mastocytoma cells (H-2^d).

For histochemical examination, 3×10^4 L-cells were cultured on coverslips in Leighton tubes. For evaluation of target cell lysis, volumes of 0.6 ml of Parker's medium containing 3×10^4 ^{51}Cr-labelled target cells (*Poulsen et al.* 1975) were cultured together with spleen cells in test tubes. Spleen cells from immunized mice or control mice were added to all cultures in amounts of 10, 30 and 100 times the number of target cells. For each mixture, five cultures were incubated at 37° C for 6 h. The quantitation of ^{51}Cr release was performed as described previously (*Poulsen et al.* 1975). Specific lysis was calculated as: 100 (^{51}Cr released with alloimmunized spleen cells)—(^{51}Cr released with spleen cells of control mice) over the net total release of ^{51}Cr (total ^{51}Cr incorporated—^{51}Cr released with spleen cells of control mice).

Histochemistry. The interaction of lymphocytes and target cells was stopped by fixation in formalin vapour for 5 min at 37° C, as previously described (*Poulsen et al.* 1975). Acid β-glucuronidase activity was demonstrated by the simultaneous capture technique of *Hayashi* (1964), using naphtol AS-BI, β-D-glucuronide (Sigma N-1875, No. 710-0730) in a final concentration of 0.14 mg/ml and pararosaniline hydrochloride (Sigma P-3750, No. 83C-2710) in a final concentration of 0.6 mg/ml. The buffer was 0.1 mol/l in acetate and adjusted to a pH of 5.2 with N-NaOH. Carboxylic ester hydrolase activity was demonstrated by the simultaneous capture technique of *Burstone* (1962), using naphtol AS acetate (Sigma N-1500, No. 54C-5066) at a final concentration of 0.1 mg/ml and diazo red RC (Sigma D-8627, No. 79B-0260) at a final concentration of 0.8 mg/ml. The phosphate buffer (0.2 mol/l) was adjusted to a pH of 6.8. The cultures were incubated in the reaction mixture for 30 min and finally placed on a slide, sealed and immediately photographed, as described previously (*Poulsen et al.* 1975).

Results

Spleen cells of C57BL/6J (H-2b) mice sensitized against L-cells of H-2k origin aggregated around cultured L-cells and showed a marked staining for β-glucuronidase activity within a six-hour incubation period (Fig. 1). A faint staining was developed in the immune spleen cells within 3 h of incubation with target cells. In contrast, spleen cells of normal mice, of iso-immunized mice or mice sensitized against the H-2d allotype were all negative or weakly positive for β-glucuronidase (Fig. 2). Target fibroblasts fixed and stained two hours after medium change without the addition of lymphocytes showed β-glucuronidase positive granules in the perinuclear area, (cf Fig 2

Staining for carboxylic ester hydrolase activity was observed only in spleen cells of allo[illegible] mice. The staining following incubation with appropriate target cells for six hours was less intense as compared with the staining for β-glucuronidase (Fig 3). Prolongation of the period of interaction of immune spleen cells with target fibroblasts for more than six hours resulted in detachment of the cells, thus eluding our observation.



Fig. 1. Spleen cells of C57BL/6J (H-2b) mice sensitized against L-cells (H-2k) and incubated for six hours on cultured L-cells in a ratio of 30 spleen cells per target cell. The naphthol AS-BI β-glucuronate—Pararosaniline method for demonstration of acid β-glucuronidase activity revealed red coloured clusters of lymphoid cells adhering to the target fibroblast indicated by white arrows. Magnification 500 times. — 25 μ.



Fig. 2. Spleen cells of unimmunized C57BL/6J (H-2b) mice incubated for six hours on cultured L-cells (H-2k) in a ratio of 30 lymphocytes per target cell. White arrows indicate granules in lymphoid cells weakly positive for β-glucuronidase activity. Black arrows indicate β-glucuronidase positive granules in a target cell. Naphthol AS-BI β-glucuronate—Pararosaniline. Magnification 500 times. 25 μ.

[illegible] specific lysis of the ^{51}Cr-labelled L-



Fig. 3. C57BL/6J spleen cells of mice sensitized against L-cells and incubated for six hours on cultured L-cells in a ratio of 30 lymphocytes per target cell. The naphthol AS acetate—Diazo red RC method revealed a faint red coloured staining for carboxylic ester hydrolase of lymphocytes adhering to target cells indicated by white arrows. Magnification 1250 times. — 10 μ.

cells exposed to immune spleen cells for six hours (five determinations) was 8 per cent, 9 per cent, and 14 per cent in the ratio of spleen cells to target cells of 10 to 1, 30 to 1, and 100 to 1 respectively.

Discussion

The present observations of appearance of staining for acid β-glucuronidase and carboxylic ester hydrolase activity in sensitized spleen cells mediating cytolysis of allogeneic fibroblasts, add further support to the suggestion that lysosomal hydrolases might have a bearing on cell-mediated target cell injury (*Poulsen et al.* 1975). It should be borne in mind that the enzymes studied, i.e. acid phosphatase, β-glucuronidase, and carboxylic ester hydrolase, are present in most tissue as isozymes (*Lundin* & *Allison* 1966). Furthermore, microsomal as well as lysosomal localization have been demonstrated (*Dingle* 1972). However, the methods of fixation in the histochemical demonstration of lysosomal enzymes and the cytochemical reactions involved, seem to result in an accentuated staining of isozymes localized to lysosomes with relative inhibition of isozymes situated elsewhere (*Dingle* 1972). Electron microscope examinations are in progress to demonstrate the subcellular localization of the activated hydrolases in sensitized lymphoid cells mediating cytolysis *in vitro*.

The present observation of appearance of staining for carboxylic ester hydrolase (esterase) activity in immune spleen cells during their lysis of target cells, may lend support to the suggestion forwarded by *Ferluga et al.* (1972) that activation of an esterase may be an early step in cytotoxic killing of target cells by immune spleen cells. This suggestion was based on the observation that organophosphorus agents which specifically and irreversibly inhibit some esterases, also inhibited cytotoxic killing of target cells by immune spleen cells.

As previously discussed (*Poulsen et al.* 1975), cultured fibroblasts can reactivate *in vivo* sensitized lymphoid cells. Thus, our observations of activation of acid hydrolases in sensitized lymphoid cells seeded on the appropriate target fibroblasts (*Poulsen et al.* 1975, and the present paper), may reflect intracellular processes occurring during *in vitro* reactivation and blast transformation. Experiments employing inhibitors of the mitosis are in progress to explore this possibility.

The authors are indebted to Dr *Helge Andersen*, Anatomy Department A, University of Copenhagen, for his valuable advice and help in histochemical methodology. The excellent technical assistance of Mrs *Kirsten Florentz* is gratefully acknowledged. This investigation was supported by *The Danish Medical Research Council* (Grants Nos 512-817, 512-1164 and 512-4045).

References: *Biberfeld, P.*: Cell. Immunol. *2*: 54-72, 1971.—*Brondz, B. D., Snegirõva, A. E., Rassulin, I. A.* & *Shamborant, O. G.*: Immunochemistry *10*: 175-189, 1973.—*Burstone, M. S.* Enzyme Histochemistry and Its Application in Study of Neoplasms. Academic Press, New York and London 1962, p. 324.—*Cerottini, J.-C.* & *Brunner, K. T.* In: Bloom, B. R. & Glade, P. (Eds): In vitro methods in cell-mediated immunology. Academic Press, New York and London 1971, p. 369.—*Cohnen, G., Douglas, S. D., König, E.* & *Brittinger, G.*: Exp. Cell Res. *80*: 297-301, 1973.—*Dingle, J. T.*: Lysosomes, a laboratory handbook. North-Holland, Amsterdam and London 1972, p 206.—*Duve, C. de.* In: Dingle, J. T. & Fell, H B. (Eds.): Lysosomes in Biology and Pathology. Vol. 1. North-Holland, Amsterdam and London 1969, p. 3.—*Ferluga, J., Asherson, G L.* & *Becker, E. L.*: Immunology *23*: 577-590, 1972.—*Hayashi, M.*: J. Histochem. Cytochem. *12*: 659 668, 1964. —*Lundin, L. G.* & *Allison, A. C* Acta chem. scand. *20*. 2579-2584, 1966.—*Poulsen, P. B., Güttler, F.* & *Jørgensen, P. N*: Acta path. microbiol. scand. Sect. C, *83*: 83-94, 1975 *Weissmann, G.* & *Dukor, P.*: Adv. Immunol. *12* 283-331, 1971.

Acta path. microbiol. scand. Sect. C, 83: 249–257, 1975

COMPARISON BETWEEN THYMUS-DEPENDENT AND THYMUS-INDEPENDENT LYMPHOCYTES AS STIMULATOR CELLS IN ALLOGENEIC MIXED LYMPHOCYTE CULTURE

PEKKA HÄYRY, SIRKKA KONTIAINEN, STIG NORDLING
and LEIF C. ANDERSSON

Third Department of Pathology, Department of Serology and Bacteriology,
University of Helsinki, and Fourth Department of Surgery, University of Helsinki Central
Hospital, SF 00290 Helsinki 29, Finland

Häyry, P., Kontiainen, S., Nordling, S. & Andersson, L. C. Comparison between thymus-dependent and thymus-independent lymphocytes as stimulator cells in allogeneic mixed lymphocyte culture. Acta path. microbiol scand. Sect. C, 83: 249–257, 1975.

Resting and activated DBA/2 T and non-T spleen lymphocytes were compared with a view to their ability to stimulate CBA spleen T lymphocytes in one-way mixed lymphocyte culture. Resting T and non-T cells were obtained by fractionation of spleen lymphocytes in preparative free flow cell electrophoresis. Activated T and non-T cells were obtained by stimulation of DBA/2 spleen cells with phytohaemagglutinin and *E. coli* lipopolysaccharide, respectively, and by fractionation of the activated cells (blasts) in one-g velocity sedimentation. Both resting and activated DBA/2 T and non-T cells induced MLC responses of the same order of magnitude, provided that the culture conditions were adjusted in such a way that both types of stimulator cells survived equally well.

Key words: Lymphocytes; thymus-dependent; thymus-independent; stimulator cells.

P. Häyry, Third Department of Pathology, University of Helsinki, SF 00290 Helsinki 29, Finland.

Received 13.i.75 Accepted 3.ii.75

Recent evidence indicates that the genes (LD genes)* coding for the antigens responsible for the proliferative response in the mixed lymphocyte culture (MLC) are linked, but that they are distinct from the genes (SD genes) coding for the "serologically detectable" antigens of the major histocompatibility complex (MHC) (6, 7, 20, 21, 30). Moreover, at least one group of the LD genes seems to be closely linked if not identical to

* Non-standard abbreviation: B = antibody forming cell precursor; FCS = foetal calf serum; Ia = immune response associated (gene); LD MHC coded antigens easily detectable by lymphocyte activation; LPS = *E coli* lipopolysaccharide MHC = major histocompatibility (gene) complex MLC = mixed lymphocyte culture, m = Mitomycin-C blocked counterpart in one way MLC SD MHC coded antigens detectable by commonl employed serological methods; non-T = thymus-independent lymphocyte; PHA = phytohaemagglutinin M T = thymus-dependent lymphocyte.

the immune response associated genes (Ia-genes) of the mouse (6, 30). By immunizing across certain recombinant mouse strains congeneic in the MHC, antisera have been produced which after adsorption presumably react with the gene products of the Ir-SS-Slp region only (12, 13, 16, 24). Some of these antisera seem to react primarily with non-T ('B')* cells (12, 24). On the assumption that these antisera also would be directed to the gene products of the LD genes, it has been suggested that only the B cells would be stimulatory in the MLC (24). Most reports so far published seem to agree with this concept (8, 9, 10, 11, 15, 22, 27). In this communication, we report that, if multiple histocompatibility barriers—including MHC, M and non-MHC barriers—in the mouse system are crossed, both resting and activated T and non-T cells are equally stimulatory in the MLC, and able to induce both the proliferative (MLC) and the cytotoxic (CML) responses. Furthermore, we report that the results obtained depend on the condition at culture employed, and that low-stimulation—at least in some cases—seems to reflect poor survival of stimulator cells in the mixed culture.

MATERIALS AND METHODS

Responder cells were derived from spleens of 6- to 8-week-old CBA/H-T6T6 mice (breeding nucleus obtained from the Jackson Laboratory, Bar Harbor, Maine). The spleen cell suspension was purified by lysing the red cells with 0.83 per cent NH_4Cl, and by partially removing adherent cells by incubation on glass (45 min) (17). The processed cell suspension contained >80 per cent lymphocytes but left a sufficient number of macrophages behind, thus still allowing an optimal proliferative response (17).

Responder T cells were further purified as follows: 25 × 10^6 CBA spleen lymphocytes, prepared as above, were passed through a Leukopak nylon wool column under conditions described by *Julius et al.* (19). The recovery after the column purification was 40 per cent. Less than 5 per cent of the passed cells were contaminated by surface Ig-positive cells, as detected by staining by FITC-conjugated goat anti-mouse Ig (a gift from Professor *A. Fagraeus*, State Serum Institute, Stockholm, Sweden). To ensure that macrophage density would be proper for the MLC response, 2 × 10^6 syngeneic peritoneal cells were added to the passed cells in ratios of 2 × 10^6 peritoneal cells per 50 × 10^6 passed lymphocytes.

Stimulator cells were obtained from spleens of 6- to 15-week-old DBA/2 mice (breeding nucleus obtained from the Jackson Laboratory). The spleen cell suspension was purified by red cell lysis, followed by iron powder plus magnetic treatment and 2 × glass incubation (17). The resulting cell population consisted of approximately 90 per cent lymphocytes and 10 per cent other cells (mainly myeloid and erythroid precursor cells), as judged on the basis of morphological criteria.

Resting T and non-T stimulator cells were obtained by fractionation in preparative cell electrophoresis as described (5). Three fractions were recovered: one of non-T cells, one of T cells, and one (intermediary fraction) of both types of cells (Fig. 1). The T and non-T fractions were contaminated by less than 5 per cent non-wanted cells according to our earlier experience (5). The fractionated cells were blocked by Mitomycin-C (Calbiochem, Los Angeles, Calif.) under conditions reported (17). Subscript "m" in the text denotes the Mitomycin-blocked counterpart.

Activated T and non-T stimulator cells were obtained from DBA/2 spleen lymphocyte cultures stimulated with *E. coli* lipopolysaccharide (LPS, a gift from Professor *G. Möller*, Karolinska Institute, Stockholm, Sweden) or with phytohaemagglutinin-M (PHA, Difco Laboratories, Detroit, Mich.) under conditions where only non-T cells were activated by the former and only T cells by the latter mitogen (5). On the third day of the mitogen culture, the cells were fractionated by one-g velocity sedimentation (2, 26) and the *blast* fraction (cells >9 μm in average cell diameter) was recovered (2). The blast fraction was contaminated less than 3 per cent by non-blastoid cells. The blasts were then blocked with Mito-

* In this paper, the designation "T" is restricted to θ-antigen carrying lymphocytes of the peripheral lymphoid organs, irrespective of the amount of the antigen on their surface. θ-positive cells are known to occupy more than 95 per cent of the cells in the high mobility region after fractionation in preparative cell electrophoresis if our usual lymphocyte processing methods for lymphocyte preparation are employed (5). The designation "non-T" (thymus-independent) is used to denote all other lymphocytes not carrying the θ-antigen. This group is recovered after electrophoretic fractionation in the low mobility region and includes, in addition to the actual antibody *forming* cell precursors ('B' cells), also other types of non-T cells characterized by diverse differently expressed surface markers such as large amount of surface immunoglobulin, receptors for C3, receptor for Fc-part of Ig, etc. (5).

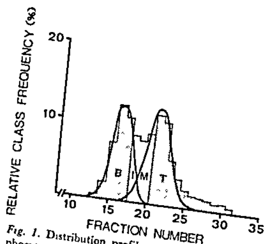


Fig. 1. Distribution profile of DBA/2 spleen lymphocytes in preparative free flow electrophoresis. High mobility cells (T cells) distribute to the right and low mobility cells (non-T cells) to the left. Gaussian distributions calculated as in our previous communications (5). Vertical lines indicate the "cuts" in the profile into non-T, intermediary (T plus non-T) and T fractions.

mycin-C and used as stimulator cells in the MLC as described (17).

Culture conditions have been described (17). 1.0×10^6 responder cells and 2.0×10^6 Mitomycin-C blocked stimulator cells in 2 ml of 5 per cent (or in some experiments 10 per cent) foetal calf serum (FCS, Flow Laboratories Ltd., Irvine, Scotland) – Eagle's minimum essential medium (Orion Pharmaceuticals, Helsinki, Finland) were cultured in 100 × 11.25 mm round bottom glass tubes (Munnerstadt Glaswahrenfabrik, Münnerstadt, Germany) in a humidified atmosphere of 5 per cent CO_2 in air. The responses were quantitated by differential counts from cytocentrifuged (Shandon Scientific Co., Ltd., London, England) May-Grunwald-Giemsa stained cell smears, and the number of cells was assessed by adding a known number (10^5) of glutaraldehyde fixed chicken red cells to the cultures prior to the preparation of the smears (17). This method enables the quantitation of the exact number of cells of any type per culture at a given time.

Cell-mediated lysis (CML) was quantitated by the ^{51}Cr-release (Sodium ^{51}Chromate, The Radiochemical Centre, Amersham, England) method in Falcon Microtest II tissue culture trays (Falcon Plastics, Los Angeles, Calif.) by employing 10,000 target cells (P-815 of DBA/2 strain), varying effector target cell ratios, a rocking platform and six-hour-exposure time as described (17). Specific ^{51}Cr-release was calculated, using the formula [(experimental – spontaneous release) : (maximal – spontaneous release)] × 100 (17).

RESULTS

One-way responses of CBA spleen lymphocytes to non-fractionated and electrophoretically fractionated DBA/2m lymphocytes are given in Table 1 (Exp. 249). T cells stimulated best (250×10^3 blasts per culture on the 6th culture day), even better than non-fractionated spleen cells (100×10^3 blasts per culture), whereas non-T cells stimulated least (25×10^3 blasts per culture on the 4th culture day). This finding was consistent in four similar experiments performed.

One explanation why T cells are superior to non-T cells as stimulators could be differences in their survival under the culture conditions used. To test this possibility, the number of surviving lymphocytes in the very same cultures was also quantitated. In accordance with our earlier findings (17), stimulator cells in non-mixed controls died quickly and were practically non-existent on the 5th day of culture (Table 1, Exp. 249). In non-mixed controls, however, T stimulator cells seemed to survive better than non-T stimulator cells.

Subsequently, the same experiment was repeated under conditions known to favour the survival of non-responding cells by increasing the concentration of FCS in the culture medium from 5 to 10 per cent (Table 1, Exp. 322). Under these conditions, the background responses were also higher. CBA spleen lymphocytes, however, now responded equally well to both T and non-T stimulator cells of DBA/2 strain (135×10^3 and 150×10^3 blasts per culture, respectively) and the responses in the mixed cultures were still well above the background responses in non-mixed controls (30–40×10^3 blasts per culture).

The very same experiment was repeated once more under conditions where highly purified T cells were used as responders. CBA spleen lymphocytes were passed through Leukopak nylon wool column, 2×10^6 syngeneic peritoneal cells per 50×10^6 lymphocytes were added to the column passed cells, and the T cells thus obtained were stimulated in the MLC by electrophoretically fractionated

TABLE 1. *CBA + DBA/2m MLC-Responses Using Various Types of DBA/2 Stimulator Cells*

Exp. No.	Responder[1]	Stimulator[2]	Response[3] 1	2	3	4	5	6	7
249[4]	CBA	DBA NF			*24*	*47*	*95*	*100*	*20*
					210	160	100	90	25
	CBA	DBA F T			*65*	*100*	*120*	*250*	*50*
					210	185	100	180	100
	CBA	DBA F IM			*30*	*65*	*74*	*35*	*30*
					204	170	110	95	55
	CBA	DBA F nT			*18*	*25*	*24*	*18*	*0*
					180	145	80	80	10
	CBA	–			*5*	*2*	*0*	*0*	
					50	20	2	0	
	–	DBA NF	*5*		*0*		*0*		
			210		40		2		
	–	DBA F T	*7*		*5*		*0*		
			300		75		10		
	–	DBA F IM	*5*		*0*		*0*		
			230		20		5		
	–	DBA F nT	*0*		*0*		*0*		
			200		0		0		
322[5]	CBA	DBA NF		*70*		*180*	*200*	*240*	*150*
				200		200	180	180	175
	CBA	DBA F T		*75*		*135*	*125*	*50*	*15*
				240		190	110	95	105
	CBA	DBA F nT		*50*		*145*	*150*	*85*	*40*
				200		100	100	105	100
	CBA	–	*12*	*35*		*30*	*40*	*40*	*24*
			250	200		190	170	155	150
	–	DBA NF		*0*					
				50					
	–	DBA F T		*0*					
				50					
	–	DBA F nT		*0*					
				40					
337[6]	CBA	DBA NF				*270*	*300*	*320*	*250*
						240	210	165	200
	CBA-T	DBA NF				*260*	*300*	*310*	
						250	225	200	
	CBA-T	DBA F T				*100*	*150*	*200*	*210*
						240	240	210	200
	CBA-T	DBA F IM				*110*	*180*	*200*	*220*
						210	200	200	175
	CBA-T	DBA F nT				*100*	*130*	*190*	*180*
						180	150	140	100
	CBA	–				*5*			
						25			
	CBA-T	–				*8*			
						75			
	–	DBA N F				*0*			
						10			
	–	DBA F T				*0*			
						10			
	–	DBA F nT				*0*			
						10			

TABLE 1. *(continued)*

Exp. No.	Re-sponder[1]	*Stimulator*[2]	Response[3] 1	2	3	4	5	6	7
255[7]	CBA	DBA ly			*110* 300	*280* 280	*300* 275	*330* 300	*210* 205
	CBA	DBA PHA-BL		*112* 280	*136* 270	*130* 170	*55* 160	*42* 128	
	CBA	DBA LPS-BL		*140* 270	*175* 240	*110* 186	*63* 155	*26* 130	*15* 80
	CBA	–	*10* 290		*8* 120		*5* 110		
	–	DBA PHA-BL	*100* 300		*20* 55				
	–	DBA LPS-BL	*380* 100		*40* 65		*0* 15		
	–	DBA ly	*5* 300		*4* 20		*0* 0		
	DBA	DBA PHA-BL			*26* 100		*0* 38		
	DBA	DBA LPS-BL			*10* 90		*0* 40		
	DBA	–			*6* 45		*0* 0		

[1] 1 × 10⁶ CBA/H-T6T6 spleen lymphocytes per culture.
[2] 2 × 10⁶ Mitomycin-C-blocked DBA/2 cells per culture.
[3] Responses quantitated as blasts per culture × 10³ (italics). The number of surviving small lymphocytes per culture (× 10³) shown below. Various culture days indicated *in Table Head*. *Mixed cultures*: mean values of duplicate determinations; non-mixed controls: single determinations only.
[4] NF = non-fractionated, T = cells from electrophoresis high mobility T-fraction, IM = cells from electrophoresis intermediary (T plus non-T) fraction, nT = cells from electrophoresis non-T fraction. For contamination rates by non-indicated cells, see Fig. 1 and the *section on Materials and Methods*. Cultures performed in 5 per cent concentration of FCS.
[5] As item 4, except that the cultures were performed in 10 per cent concentration of FCS.
[6] As item 4, except that the cultures were performed in 10 per cent concentration of FCS. CBA-T = Leukopak nylon wool purified CBA spleen T cells.
[7] PHA-BL = blast cells from PHA-stimulated cultures, LPS-BL = blast cells from *E. coli* LPS stimulated cultures. Fractionation of mitogen stimulated cultures performed by one-g velocity sedimentation. For purity controls see the section on Materials and Methods.

DBA/2m spleen lymphocytes. Table 1 (Exp. 337) and Fig. 2 give the results of two experiments of this kind. In growth supporting 10 per cent concentration of FCS CBA-T cells responded equally well to both T and non-T cells of the DBA/2 strain.

We also compared activated T and non-T cells (blasts) with a view to their ability to stimulate in the MLC. DBA/2 spleen lymphocytes were stimulated by *E. coli* LPS (a non-T cell mitogen) or PHA (a T cell mitogen). On the 3rd day of culture, the blasts were recovered by one-g velocity sedimentation, treated with Mitomycin-C, and used as stimulators in CBA + DBA/2m MLC.

Responses of similar magnitude were recorded in the MLC, regardless of whether PHA or LPS blasts were used as stimulator cells (Table 1, Exp. 255). DBA blasts of either type did not induce any detectable syngeneic MLC response under these conditions (Table 1, Exp. 255).

Whether T and non-T cells were able not only to induce the blast response in the MLC but also to trigger the cell-mediated lysis (CML), was further tested by employing

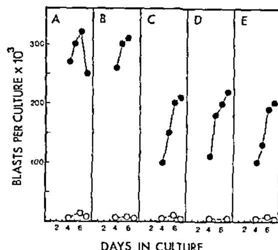


Fig. 2. MLC-responses (closed circles) of non-fractionated CBA spleen lymphocytes (A) and Leukopak column fractionated CBA spleen T lymphocytes (B) to DBA/2m spleen cells, and column fractionated CBA spleen T cells to electrophoretically fractionated DBA/2m T (C), intermediary (D) and non-T (E) lymphocytes. Mean values of background responses indicated by open circles and dashed line.

resting T and non-T cells as stimulators. CBA T lymphocytes prepared by the Leukopak nylon wool column were stimulated by electrophoretically fractionated DBA/2 spleen T and non-T lymphocytes as above. On the 8th day of culture, the cultures were harvested and tested for CML to relevant allogeneic target cells (P-815 of DBA/2 strain). The results of the two experiments performed are given in Fig. 3. If the MLC-priming was performed in the presence of a supporting 10 per cent concentration of FCS, resting T cells and resting non-T cells would be equally good to stimulate allogeneic T cells to killer cells.

DISCUSSION

Physical methods of fractionation were employed in the preparation of T and non-T cells. These methods may be regarded superior to methods using anti-T and anti-B cell sera plus complement if non-wanted cell populations in experiments of this type are to be eliminated. This is since physical preparation methods exclude the possibility of deposition of antigen-antibody complexes on the surface of the wanted cell population which complexes in turn might interfere with their ability to stimulate. The methods employed also give a high degree of purity. With appropriate cuts in the electrophoresis profile (Fig. 1), the contamination rates in resting T and non-T fractions by non-wanted cells is less than 5 per cent (5). When the one-g velocity sedimentation profile is cut at 9 µm of average cell diameter, the activated cells (blasts) are contaminated by less than 3 per cent with non-activated cells (lymphocytes) (2). Furthermore, the MLC responses were performed under conditions where only T cells are responsive (3, 4), or highly purified T cells were as responders (19). Thus, it may be concluded that the responses described in this paper most likely represent CBA T cell responses to DBA/2 T and non-T cells.

The CBA T cell response to DBA/2 T or non-T cells seems to be affected by the conditions of culture employed. Under conditions in which stimulator T cells survive better than non-T cells, T cells seem to be superior to non-T cells in triggering the response. Under conditions where the survival of stimulator T and non-T cells is equal (a high concentration of supporting serum), both types of cells seem to stimulate equally well. Activated T and non-T cells survived equally well regardless of the concentration of serum in the medium. If activated DBA/2 T and non-T cells were employed as stimulator cells, responses of the same order of magnitude were observed. Thus it may be concluded that both resting and activated DBA/2 spleen T and non-T lymphocytes are able to trigger CBA spleen T cells in the MLC.

In previous studies where the MLC assay has been used to localize the stimulating antigens on T vs. non-T cell surface, five different approaches have been employed: (1), lymph node or spleen cells of rats or mice thymectomized shortly after birth (25) or deprived from T cells (15) have been compared with those of non-treated animals; (2), cells from diverse lymphoid organs known to

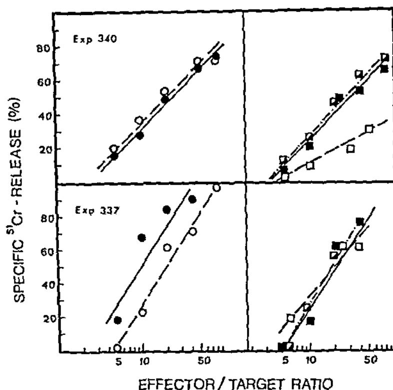


Fig. 3. P-815 (of DBA/2 strain) target cell lysis by CBA + DBA/2m MLC-primed cells. Two separate experiments are described. Left side: open circles = CBA spleen lymphocytes primed in the MLC with non-fractionated DBA/2m spleen cells; closed circles = Leukopak column purified CBA spleen T lymphocytes primed in the MLC with DBA/2m spleen cells. Right side: Leukopak column fractionated CBA spleen T lymphocytes primed in the MLC by electrophoretically fractionated DBA/2 T (closed squares), intermediary (half-closed squares) and non-T (open squares) spleen lymphocytes. Exp. 340: 5 per cent FCS; Exp. 337: 10 per cent FCS. The results is expressed as specific ^{51}Cr release in varying effector/target cell ratios by employing 6-hour-exposure time. Background release less than 12 per cent of maximal.

be enriched by T or non-T cells have been tested (11); (3). lymphoid cells enriched by T or non-T cells by certain physical methods of fractionation have been employed (8, 9); (4). homozygous (nu/nu) and heterozygous (nu/+) litter mates of nude mice with congeneic thymus aplacia have been used (10), and (5). the non-wanted cell population has been removed by anti-T or anti-B cell sera plus complement (27). In addition to this, a report is available according to which the cells in a human subject with congenital thymic aplasia, carried a normal set of HL-A (SD) antigens but failed to stimulate in the MLC (14). Three different types of results have been obtained: T cells being better stimulators than non-T cells (14, 25), T cells being equally good as non-T cells (8), and T cells being inferior to non-T cells (9, 10, 11, 15, 22, 27).

In these studies, a large number of different strain combinations have been employed, including stimulator cells from several different mouse strains. It is noteworthy, however, that the responses in the MLC have always been quantitated by incorporation of a radioactive nucleotide only, and controls of the survival of the stimulator cells in the various culture systems have not been included. Since the survival of stimulator cells in the cultures may strongly affect the result, it is not possible to make direct compari-

sons between this study and those reported previously.

CBA response to DBA/2m may be affected by the following known histoincompatibilities: MHC (H-2) incompatibility (H-2^k/H-2^d), M-locus incompatibility (M2/M1) and multiple non-MHC (non-H-2) incompatibilities. Although the MLC-response of human lymphocytes seems to be directed to the MHC incompatibility only (29), this may not be so in the case of the mouse. Incompatibility in the M-locus often results in a very strong stimulation (1) although cytotoxic lymphocytes do not seem to be generated to lymphoblastoid target cells (1), and multiple and even well defined non-H-2 incompatibilities may be MLC stimulatory as well (18, 28). Thus the combination employed in our study may be useful as a "screening" test since several histocompatibility differences are covered at the same time. However, these experiments fail to provide any information about antigenic specificities coded by single histocompatibility loci.

Lonai & McDevitt (23) have recently studied the ability of T and B lymph node cells from congeneic Ia-recombinant mouse strains to stimulate in one-way MLC. In three out of five combinations studied, both T and B cells were stimulatory whereas in one combination (differing at IaC only), only T cells stimulated. This finding suggests that some of the genes in the Ia-region may be expressed on both T and B cells, whereas others may be expressed on either cell type only.

Recently, antisera have been produced by reciprocal immunization between mouse strains differing in the IR-SS-Slp region of the MHC only. One of these antisera (the anti-Ir-1.1. serum) apparently reacts with T cells only (16), a second antiserum (the anti-Lna serum) reacts with both T and non-T cells, but at high dilution only with T cells (12), while the third antiserum seems to be reactive with non-T cells only (24). Whether the antigens detected by these antisera would be associated with, or identical to the MLC-stimulating antigens is still an open question. Blocking experiments with these antisera, and MLC responses with MHC-congeneic strain combinations in which T or non-T cells have been employed as stimulators would possibly elucidate this interrelationship.

We thank Miss *Monica Schoultz* and Mrs. *Hilkka Sokura* for technical assistance. This study was supported by grants and research contracts from the *Sigrid Jusélius Foundation*, the *National Research Council for Medical Sciences*, the *Association of Finnish Life Insurance Companies*, Helsinki, and the *Délégation Générale à la Recherche Scientifique et Technique* (D.G.R.S.T.), Paris.

REFERENCES

1. *Abbasi, K. & Festenstein, H.:* Antigenic strength investigated by cell-mediated lympholysis in mice. Eur. J. Immunol *3:* 430–435, 1973.
2. *Andersson, L. C. & Häyry, P.:* Generation of T memory cells in one-way mixed lymphocyte culture. I. Selective recovery of activated cells and their reversion to 'secondary' lymphocytes. Scand. J. Immunol. *3:* 461–469, 1974.
3. *Andersson, L. C., Nordling, S. & Häyry, P.:* Allograft immunity in vitro. VI. Autonomy of T lymphocytes in target cell destruction. Scand. J. Immunol. *2:* 107–113, 1973.
4. *Andersson, L. C., Nordling, S & Häyry, P.:* Proliferation of T and B cells in mixed lymphocyte cultures. J. exp. Med. *138:* 324–329, 1973.
5. *Andersson, L. C., Nordling, S. & Häyry, P.:* Fractionation of mouse T and B lymphocytes by preparative cell electrophoresis. Efficiency of the method. Cell. Immunol. *8:* 235–248, 1973.
6. *Bach, F. H., Widmer, M. B., Bach, M. L. & Klein, J.:* Serologically defined and lymphocyte defined components of the major histocompatibility complex in the mouse. J. exp. Med. *136:* 1430–1444, 1972.
7. *Bach, M. L., Widmer, M. B., Bach, F. H. & Klein, J.:* Mixed leukocyte cultures and immune response region disparity. Transplant. Proc *5:* 369–375, 1973.
8. *von Boehmer, H.:* Separation of T and B lymphocytes and their role in the mixed lymphocyte reaction. J. Immunol. *112:* 70–78, 1974.
9. *von Boehmer, H.:* Selective stimulation by B lymphocytes in the syngeneic mixed lymphocyte reaction. Eur. J. Immunol *4:* 105–110, 1974.

0. *Cheers, C.* & *Sprent, J.*: B lymphocytes as stimulators of a mixed lymphocyte reaction. Transplantation *15:* 336–337, 1973.
1. *Click, R. E.*: Immune responses in vitro. IX. Absence of the mixed leukocyte stimulatory, M-locus product from thymus cells. J. exp. Med. *139:* 1628–1634, 1974.
2. *David, C. S., Frelinger, J. A.* & *Shreffler, D. C.*: New lymphocyte antigens controlled by the Ir-IgG region of the H-2 gene complex. Transplantation, *17:* 122–125, 1974.
13. *David, C. S.* & *Shreffler, D. C.*: Lymphocyte antigens controlled by the Ir region of the mouse H-2 complex. Detection of new specificities with anti-H-2 reagents. Transplantation *17:* 462–469, 1974.
14. *Eijsvoogel, V. P., Schellekens, P. T. A., Breuer-Vriesendorp, B., Konig, L., Koch, C., van Leuten, A. A. D.* & *van Rood, J. J.*: Mixed lymphocyte cultures and HL-A. Transpl. Proc. *3:* 85–88, 1971.
15. *Harrison, M. R.*: Thymus independent stimulator cells in the mixed lymphocyte reaction. J. Immunol. *111:* 1270–1273, 1973.
16. *Hauptfeld, V., Klein, D.* & *Klein, J.*: Serological identification of an Ir-region product. Science *181:* 167–169, 1973.
17. *Häyry, P., Andersson, L. C., Nordling, S.* & *Virolainen, M.*: Allograft response in vitro. Transplant. Rev. *12:* 91–140, 1972.
18. *Häyry, P.* & *Defendi, V.*: Allograft immunity in vitro. III. Induction of deoxyribonucleic acid synthesis in mixed cultures of mouse peripheral lymphocytes from inbred strains differing at non-H-2 loci. Transplantation *9:* 410–416, 1970.
19. *Julius, M. H., Simpson, E.* & *Hertzenberg, L. A.*: A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur. J. Immunol. *3:* 645–649, 1973.
20. *Klein, J.* & *Park, J. M.*: Graft-versus-host reaction across different regions of the H-2 complex of the mouse. J. exp. Med. *137:* 1213–1255, 1973.
21. *Livnat, S., Klein, J.* & *Bach, F. H.*: Graft versus host reaction in strains of mice identical for H-2K and H-2D antigens. Nature New Biol. *243:* 42–44, 1973.
22. *Lohrmann, H.-P., Novikovs, L. D.* & *Graw, R. G.*: Stimulatory capacity of human T and B lymphocytes in mixed leukocyte culture. Nature *250:* 144–146, 1974.
23. *Lonai, P.* & *McDevitt, H. O.*: I region genes are expressed on T and B lymphocytes. Studies on the mixed lymphocyte reaction (MLR) J. exp. Med. *140:* 1317–1323, 1974.
24. *Lozner, E. C., Sachs, D. H., Shearer, G. M.* & *Terry, W. D.*: B-cell alloantigens determined by the H-2 linked Ir region are associated with mixed lymphocyte stimulation. Science *186:* 757–759, 1974.
25. *McLaurin, B. P.*: Thymus origin of lymphocytes reacting and stimulating reaction in mixed lymphocyte cultures—studies in the rat. Clin. exp. Immunol. *10:* 649–659, 1972.
26. *Miller, R. G.* & *Phillips, R. A.*: Separation of cells by velocity sedimentation. J. Cell Physiol. *73:* 191–202, 1969.
27. *Plate, J. M. D.* & *McKenzie, I. F. C.*: 'B'-cell stimulation of allogeneic T-cell proliferation in mixed lymphocyte cultures. Nature New Biol. *245:* 247–249, 1973.
28. *Rychlíková, M.* & *Iványi, P.*: Mixed lymphocyte cultures and histocompatibility antigens in mice. Folia biol. (Praha) *15:* 126–135, 1969.
29. *Sørensen, S. F.*: The mixed lymphocyte culture interaction. Acta path. microbiol. scand. Sect. B, suppl. *230:* 1–82, 1972.
30. *Vives, J., Meo, T., Miggiano, V.* & *Shreffler, D.*: A major role for the Ir-1 region of the mouse H-2 complex in the mixed leukocyte reaction. Transplant. Proc. *5:* 377–381, 1973.

Acta path. microbiol. scand. Sect. C, *83*: 258–264, 1975

MORPHOLOGICAL AND HAEMATOLOGICAL STUDIES OF THE *IN VIVO* EFFECT OF PHYTOHAEMAGGLUTININ, POKEWEED MITOGEN, AND BACILLUS CALMETTE-GUÉRIN ON DBA/2 MICE

JORGEN REM, MOGENS KROGH JENSEN and JENS ERLAND OLSEN

The Fibiger Laboratory, 2100 Copenhagen, Denmark, Division of Haematology, Department of Medicine B, Aalborg Hospital, 9100 Aalborg, Denmark, and Department of Pathology, Centralsygehuset, 4800 Nykobing Falster, Denmark

Rem, J., Jensen, M. K. & Olsen, J. E. Morphological and haematological studies of the *in vivo* effect of phytohaemagglutinin, pokeweed mitogen, and bacillus Calmette-Guérin on DBA/2 mice. Acta path. microbiol. scand. Sect. C, *83*: 258–264, 1975.

Intraperitoneal injection of either PHA, PWM, or BCG in DBA/2 mice had no effect on the haemoglobin concentration and leucocyte count. A transformation of the lymphocytes of the peripheral blood to large blast-like DNA-synthesizing cells—as seen *in vitro*—did not occur. Repeated injections of BCG produced a displacement of the blood differential count towards a higher percentage of granulocytes. Both PHA and PWM produced a significant increase in the spleen weight, corresponding to a significant enlargement of the germinal centres and an increased cellularity of the red pulp with large pyroninophilic blast-like cells. The lymph nodes were characterized by partial disorganization of the follicular structure followed by enlarged germinal centres and increased cellularity, partly consisting of blast-like cells. A single injection of BCG did not produce any significant changes in the spleen and lymph nodes. In contrast to this, administration of BCG twice at an interval of two weeks caused a significant increase in the spleen weight, accompanied by morphological alterations in the spleen and lymph nodes, essentially similar to those brought about by PHA and PWM. The bone marrow and thymus remained unaffected by PHA, PWM, and BCG.

Key words: Phytohaemagglutinin; pokeweed mitogen; bacillus Calmette-Guérin; morphology, haematology; mice.

Jorgen Rem, The Fibiger Laboratory, Ndr. Frihavnsgade 70, DK-2100 Copenhagen, Denmark.

Received 31.vii.74 Accepted 6.i.75

The phytomitogens phytohaemagglutinin (PHA) and pokeweed mitogen (PWM) have been shown to induce blastic transformation of peripheral blood lymphocytes *in vitro* (*Nowell 1960, Farnes et al. 1964*) and, if administered to rodents *in vivo*, they produce morphological changes in the lymphoid tissues (*Gamble 1966, Hartveit 1970, Paulli Jorgensen et al.* 1972, *Zazula & Fikrig* 1970). In addition, it has been reported that accidental systemic exposure of man to PWM results in the appearance of large immature lymphocytes in the peripheral blood for up

to two weeks after exposure (*Barker et al.* 1966). Furthermore, evidence has been presented that both PHA and PWM may exert radioprotection of mice lethally irradiated (*Jennings & Oated* 1967, *Stefani* 1970). In mice treated with near-lethal doses of rubidomycin, however, PHA and PWM both failed to exert any protection against the toxic side effects of this agent, whereas its toxicity was enhanced if administered in combination with PWM (*Krogh Jensen & Rem* 1973). In addition to this, a number of animal experiments indicate that phytomitogens may have an antineoplastic effect (*Haase et al.* 1968, *Robinson & Mekori* 1971).

Bacillus Calmette-Guérin (BCG) has been shown to induce a transformation in lymphocyte cultures from sensitized subjects similar to the blastic transformation obtained by PHA (*Pearmain et al.* 1963). Moreover, *in vivo* studies of the effect of BCG in relation to whole-body irradiation of mice have demonstrated that this agent exerts a significant protection against lethal irradiation for up to several weeks after a single intravenous injection (*Balner et al.* 1961). Finally, it has been shown in a number of animal experiments that the growth of various transplanted tumours can be retarded by treatment with BCG (*Mathé et al.* 1969) and, during the last few years, encouraging, but so far non-conclusive, results have appeared according to which BCG may be a possible non-specific immune stimulant in the treatment of human leukaemia (*Crowther et al.* 1973).

Much of the information about the effect of PHA and PWM has been achieved by means of different commercial mitogens, different dose-schedules, and different animal species. Therefore, only little information has been provided by comparative studies of the effect of PHA and PWM on the morphology of the lymphatic system *in vivo*, and as far as we know, no studies of the morphology of the mouse lymphoid system are available. Moreover, on the basis of the above mentioned data it cannot be doubted that phytomitogens may be of clinical value in the future management of haematological and neoplastic disorders.

Accordingly, the present study, was undertaken in order to compare and further elucidate the effect of PHA, PWM, and BCG on the mouse lymphoid system *in vivo*.

MATERIAL AND METHODS

Adult inbred DBA/2 male mice, 8 to 12 weeks old, of an average weight of 25 g, were used.

PWM (Gibco, cat ≠ 536) and PHA (purified phytohaemagglutinin, Burroughs & Wellcome, MR 68), obtained frezee-dried in vials, were reconstituted with sterile water and injected intraperitoneally at a strength of 1 mg per ml and a dosage of 10 mg per kg body weight immediately after reconstitution. BCG* was obtained in ampoules containing 6 million attenuated living, freeze-dried tubercle bacilli, (3.75 mg protein). Before use, the content of each ampoule was dissolved to a volume of 5 ml and administered by the intraperitoneal route in dosages of 5 ml per kg body weight.

Three groups of mice (9 in each) received a single injection of either PHA, PWM or BCG, whereas a fourth group of 9 mice was given two injections of BCG at intervals of two weeks. A control group of 9 mice was injected intraperitoneally with 10 ml saline per kg body weight, but was otherwise treated as described below.

On days 3, 7, and 14 after the last injection, three mice from each group were sacrificed after whole body weights had been determined. Two ml of blood from the neck of each mouse was collected in heparinized tubes One micro-Ci of ^{3}H-thymidine (1.9 Ci/Mmol, Schwarz Bio Research, Inc.) was added to each specimen which was incubated for one hour at 37° C. Blood smears were made; the slides were fixed in absolute methanol and processed for autoradiography, using Kodak NTB-2 emulsion. After exposure for one week, the slides were developed and stained with Giemsa at pH 5.50 Differential counts of five hundred cells were obtained from each mouse. A lymph node from the inguinal region, the liver, spleen, and thymus were removed and fixed in 5 per cent formalin for histological study. Before fixation of the spleens the spleen weight was determined for calculation of the spleen weight/body weight ratio. Sections were stained with haematoxylin and eosin (H-E), methylgreen pyronine, toluidine blue, and Giemsa. The left femur was removed, the upper and lower epiphyses were cut off and

* Kindly supplied by Statens Seruminstitut, Copenhagen.

the marrow flushed out using a syringe and fine needle for smears. The slides were fixed in absolute methanol and stained with Giemsa at pH 5.50.

In other groups similarly treated with PHA, PWM, BCG, and saline, 10 to 30 mice from each group were sacrificed on days 3, 7, and 14 after the last treatment. Whole body weights and spleen weights were determined and the spleen weight/body weight ratios calculated as described above. Blood was drawn from the orbital sinus using 20 microlitre pipettes for determination of the haemoglobin concentration and leucocyte count, as described elsewhere (*Norgaard-Pedersen & Siggaard-Andersen 1970*).

As the obtained values were non-parametrically distributed, the statistical calculations were done according to the non-parametric Man Whitney rank sum test.

RESULTS

1. Effect on the Haemoglobin Concentration

A considerable variation in the haemoglobin concentration, within as well as between the various groups, was found in this study. The differences, however, were not statistically significant.

2. Effect on the Leucocyte Count

On days 7 and 14 after treatment with PHA, PWM, or BCG there was a slight relative increase in the leucocyte count. This increase, however, was only statistically significant on day 7 in the group treated once with BCG ($p = 0.02$).

3. Effect on the Morphology of the Lymphocytes of the Peripheral Blood

As seen from Table 1, the number of DNA-synthesizing cells in groups treated with PHA, PWM, or BCG did not differ from that in the controls. Furthermore, we were not able to demonstrate any blast-transformation of the peripheral blood lymphocytes.

The blood differential counts did not show any changes after treatment with PHA, PWM, or BCG given as a single injection

TABLE 1. *Proportion of DNA-Synthesizing Mononuclear Cells and Differential Count in Peripheral Blood From Mice Treated With PHA, PWM, and BCG*

Group	Days after treatment	No. of mice	Per cent labelled cells	Per cent lymphocytes	Per cent granulocytes
	3	3	0.1	73	27
I	7	3	0.6	71	29
	14	3	0	64	36
	3	3	0.7	69	31
II	7	3	0	79	21
	14	3	0	71	29
	3	3	0.1	66	34
III	7	3	0.1	76	24
	14	3	0.1	80	20
	3	3	0.1	54	46
IV	7	3	0	54	46
	14	3	0.1	54	46
	3	3	0.5	73	27
control	7	3	0	63	37
	14	3	0.1	65	35

Groups I, II, and III were treated with 10 mg PHA, 10 mg PWM, and 5 ml BCG, respectively, per kg body weight on day 0. Group IV was treated with 5 ml BCG per kg body weight both on day—14 and on day 0. The control group was treated with saline. All the agents were administered by the intraperitoneal route. 500 cells were obtained from each mouse. Each of the percentage values in the table represents the average of three mice.

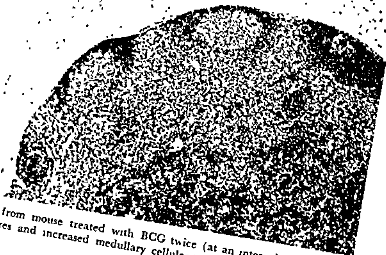


Fig. 1. Lymph node from mouse treated with BCG twice (at an interval of two weeks), showing prominent germinal centres and increased medullary cellularity on day 14 after the last treatment. (H-E, × 56).

on day 0, compared to the control group. In contrast to this, however, administration of BCG twice at an interval of two weeks caused a significant displacement of the differential count towards a higher percentage of granulocytes.

4. *Effect on Bone Marrow Cells*

No quantitative or morphological changes were seen within the various cell types of the marrow after treatment with PHA, PWM, or BCG.

5. *Effect on the Lymph Node*

In most of the lymph nodes, PWM produced a slight oedema and a moderate proliferation of the reticulum cells. After 3 days, the follicles were partly disorganized. On day 7, however, prominent germinal centres were present and, in the medullary region, a marked occurrence of blast-like cells was found. After 14 days there was some decrease in the cellularity of the medulla, but the follicles remained large and prominent.

Three days after injection of PHA, the lymph nodes were characterized by oedema and disorganization of the follicular structure. Only a few germinal centres were prominent, but without enlargement and scattered blast-like cells were found in the sinusoids. On day 7, the germinal centres were large and prominent, tending towards confluence. The paracortical and medullary sinusoids contained many small lymphocytes interspersed with a slightly increased number of blast-like cells. After 14 days, several of the germinal centres were still prominent, but slowly regressing with an overall tendency towards normalization of the follicular structure which had been rebuilt in several areas of the lymph nodes.

A single injection of BCG did not produce any significant morphological alterations in the lymph nodes after three days. On day 7, however, there was a slight proliferation of the reticulum cells accompanied by a tendency towards disorganization of the follicular structure. After 14 days, the lymph nodes were slightly oedematous; the germinal centres, however, were still preserved and of normal size, while the sinusoids contained a slightly increased number of small lymphocytes.

the marrow flushed out using a syringe and fine needle for smears. The slides were fixed in absolute methanol and stained with Giemsa at pH 5.50.

In other groups similarly treated with PHA, PWM, BCG, and saline, 10 to 30 mice from each group were sacrificed on days 3, 7, and 14 after the last treatment. Whole body weights and spleen weights were determined and the spleen weight/body weight ratios calculated as described above. Blood was drawn from the orbital sinus using 20 microlitre pipettes for determination of the haemoglobin concentration and leucocyte count, as described elsewhere (*Nørgaard-Pedersen* & *Siggaard-Andersen* 1970).

As the obtained values were non-parametrically distributed, the statistical calculations were done according to the non-parametric Man Whitney rank sum test.

RESULTS

1. Effect on the Haemoglobin Concentration

A considerable variation in the haemoglobin concentration, within as well as between the various groups, was found in this study. The differences, however, were not statistically significant.

2. Effect on the Leucocyte Count

On days 7 and 14 after treatment with PHA, PWM, or BCG there was a slight relative increase in the leucocyte count. This increase, however, was only statistically significant on day 7 in the group treated once with BCG ($p = 0.02$).

3. Effect on the Morphology of the Lymphocytes of the Peripheral Blood

As seen from Table 1, the number of DNA-synthesizing cells in groups treated with PHA, PWM, or BCG did not differ from that in the controls. Furthermore, we were not able to demonstrate any blast-transformation of the peripheral blood lymphocytes.

The blood differential counts did not show any changes after treatment with PHA, PWM, or BCG given as a single injection

TABLE 1. *Proportion of DNA-Synthesizing Mononuclear Cells and Differential Count in Peripheral Blood From Mice Treated With PHA, PWM, and BCG*

Group	Days after treatment	No. of mice	Per cent labelled cells	Per cent lymphocytes	Per cent granulocytes
I	3	3	0.1	73	27
	7	3	0.6	71	29
	14	3	0	64	36
II	3	3	0.7	69	31
	7	3	0	79	21
	14	3	0	71	29
III	3	3	0.1	66	34
	7	3	0.1	76	24
	14	3	0.1	80	20
IV	3	3	0.1	54	46
	7	3	0	54	46
	14	3	0.1	54	46
control	3	3	0.5	73	27
	7	3	0	63	37
	14	3	0.1	65	35

Groups I, II, and III were treated with 10 mg PHA, 10 mg PWM, and 5 ml BCG, respectively, per kg body weight on day 0. Group IV was treated with 5 ml BCG per kg body weight both on day −14 and on day 0. The control group was treated with saline. All the agents were administered by the intraperitoneal route. 500 cells were obtained from each mouse. Each of the percentage values in the table represents the average of three mice.

to an antigen capable of stimulating a wide range of otherwise specific immunological clones.

The results of the present study, therefore, strongly indicate further experiments with the various mitogens in laboratory animals in order to clarify their *in vivo* action, and especially in order to compare the actions of mitogens such as PHA and PWM, which are commonly accepted to be purely mitogenic, to that of immunological acting agents such as BCG.

REFEERENCES

Astaldi, G., Burgio, G. R., Genova, R., Curtoni, E. & Giraudo, L. C.: Histological skin reaction to intradermal inoculations of pokeweed in humans. J. reticuloendoth. Soc. *7:* 126–139, 1970.

Balner, H., Old, L. J. & Clarke, D. A.: Reduction of X-ray lethality in mice by agents affecting the reticuloendothelial system. Radiat. Res. *15:* 836–849, 1961.

Barker, B. E., Farnes, P. & LaMarche, P. H.: Peripheral blood plasmacytosis following systemic exposure to phytolacca americana (pokeweed). Pediatrics *38:* 490–493, 1966.

Crowther, D., Powles, R. L., Bateman, C. J. T., Beard, M. E. J., Gauci, C. L., Wrigley, P. F. M., Malpas, J. S., Hamilton Fairley, G. & Bodley Scott, R.: Management of adult acute myelogenous leukaemia. Britt. Med. J. *1:* 131–137, 1973.

Farnes, P., Barker, B. E., Brownhill, L. E. & Fanger, H.: Mitogenic activity in phytolacca americana. Lancet *ii:* 1100–1101, 1964.

Gamble, C. N.: The effects of phytohemagglutinin on mouse spleen cells in vivo. Blood *28:* 175–187, 1966.

Haase, A. T., Kasel, J. A. & Chessin, L. N.: Adenovirus type 12 oncogenicity in newborn hamsters: The effect of pokeweed mitogen. J. Immunol. *101,* 806–807, 1968.

Hartveit, F.: Changes in the lymphoid system following the intravenous injection of phytohaemagglutinin in mice. Acta path. microbiol. scand Sect. A, *78:* 525–531, 1970.

Janossy, G. & Greaves, M. F.: Lymphocyte activation. (Response of T and B lymphocytes to phytomitogens). Clin. exp. Immunol. *9:* 483–498, 1971.

Jennings, J. F. & Oates, C. M.: The effect of phytohaemagglutinin on the immune response in vivo. Clin. exp. Immunol. *2:* 445–453, 1967.

Krogh Jensen, M. & Rem, J.: Influence of pokeweed mitogen, phytohemagglutinin, and bacillus Calmette-Guérin on the toxicity of rubidomycin on DBA/2 mice. Proc. Soc. Exp. Biol. Med. *144:* 639–643, 1973.

Lozzio, B. B. & Comas, F. V.: Biological effects of pokeweed mitogen. Int. Arch. Allergy *36:* 266–281, 1969.

Lozzio, B. B., Machado, E. A. & Chernoff, I. A.: Functional and cellular alterations produced by phytohaemagglutinin. Acta haemat. *41:* 349–358, 1969.

Mathé, G., Pouillart, P. & Lapeyraque, F.: Active immunotherapy of L1210 leukaemia applied after the graft of tumor cells. Brit. J. Cancer *23:* 814–824, 1969.

Nowell, P. C.: Phytohemagglutinin: An initiator of mitosis in cultures of normal human leukocytes. Cancer Res. *20:* 462–466, 1960.

Nørgaard-Pedersen, B. & Siggaard-Andersen, O.: A method for microscopic counting of thrombocytes and leukocytes, with special emphasis on the use of capillary blood Ugeskr. Læg. *132:* 1228–1232, 1970.

Paulli Jørgensen, H., Krogh Jensen, M. & Olsen, J. E.: Studies on the in vivo effect of pokeweed mitogen (PWM) on the lymphoid tissue of the rat. Acta path. microbiol. scand. Sect. A, *80:* 367–373, 1972.

Pearmain, G., Lycette, R. R. & Fitzgerald, P. H.: Tuberculin-induced mitosis in peripheral blood leukocytes. Lancet *1:* 637–638, 1963.

Robinson, E. & Mekori, T.: Studies on the effect of phytohemagglutinin on ascites tumor in mice. Israel J. med. Sci. *7:* 83–89, 1971.

Stefani, S.: Radioprotective effect of phytohemagglutinin in mice. Experientia *26* 80–81, 1970.

Stockman, G. D, Gallagher, M. T, Heim, L. R. South, M. A. & Trentin, J. J. Differential stimulation of mouse lymphoid cells by phytohemagglutinin and pokeweed mitogen. Proc. Soc. Exp. Biol. Med. *136:* 980–982, 1971.

Zazula, B. M. & Fikrig, S. M In vivo effects of pokeweed mitogen on mouse spleen cells. Proc. Soc. Exp. Biol. Med. *133·* 1088–1092, 1970

(35) and the presence of Ig-containing inclusions in the leukocytes (5, 39).

In recent years, increasing evidence has been presented that cryoglobulins (CG) isolated from human sera represent cold-insoluble immune complexes (3, 18, 28). The most direct proof stems from the study of CG in animals with experimental serum sickness, where the isolated CG was greatly enriched with the immunizing antigen and specific antibody (12). Recently, CG containing immunoglobulins and deoxyribonucleic acid were demonstrated in a large percentage of SF from RA patients (7, 21), and antinuclear factors (ANF) and rheumatoid factors were found to participate in the cryoprecipitable material (7, 21, 31, 39). A considerable portion of the complement-fixing activity in SF was cryoprecipitable (27). It is thus conceivable that SF CG in RA may play a role in the chronic inflammatory processes by induction of local type III-reactions (10, 30, 37).

In an attempt to evaluate the possible pathogenetic significance of granulocyte-specific antinuclear factors (GS-ANF), so commonly found in RA sera and SF (9, 32), we have investigated CG from rheumatoid SF for occurrence of GS-ANF. Another aim of this study was to elucidate whether these proteins really represent immune complexes or mere cryofibrinogens containing trapped immunoglobulins from the SF. We have therefore done approximated quantitations of IgG, IgA and IgM as well as total protein content of the CG in RA and osteo-arthrosis (OA) patients Fibrinogen-antigenic material, C1q, C4 and C3 has been looked for by double immunodiffusion.

The data obtained are compatible with the notion that SF CG in RA, at least in part, represent immune complexes containing GS-ANF and sometimes organ-nonspecific antinuclear factors (ON-ANF), both of which may be significantly enriched in the CG compared to the corresponding SF.

MATERIALS AND METHODS

77 patients fulfilling the criteria of the American Rheumatism Association for classical and definite RA (26) and 24 patients fulfilling generally accepted criteria for osteo-arthrosis including normal erythrocyte sedimentation rate and serum electrophoresis and typical clinical and radiological features, all admitted to the Department of Rheumatology, Frederiksberg Hospital, Copenhagen, were selected for the study solely on the basis of a presence of sufficient amounts of available SF. The age and sex distribution of the patients is shown in Table 1.

SF was aspirated under sterile conditions into tubes containing EDTA in a final concentration of about 0.01 mMol/ml. Cells were removed by centrifugation at 400 × g for ten minutes, and the supernatant was incubated with testicular hyaluronidase (Penetrase, LEO, Copenhagen) 150 IU/ml SF for thirty minutes at +37° C. The SF was incubated at +4° C for 24 hours, and cryoprecipitable material was pelleted at 1000 × g for ten minutes. The supernatant SF was stored at −20° C. The CG was then resuspended and washed four times in ice cold phosphate buffered saline pH 7.2 (PBS) in an attempt to remove nonspecifically trapped proteins from the precipitate, and the CG was redissolved in a small volume of PBS at +37° C. Insoluble material, equally present in RA and OA specimens, was removed by centrifugation

TABLE 1. *Age and Sex Distribution of All Patients*

	Cryoglobulin isolated*	No.	Mean	Range	Men/women
Rheum. arthritis	+	42	64.5	32–83	12/30
	—	35	66.3	43–87	9/26
Osteo-arthrosis	+	18	75.4	37–88	8/10
	—	6	74.8	64–82	0/ 6

* Sufficient amount of cryoglobulin in the synovial fluid to allow isolation, washing, measurement of protein, IgG, IgA and IgM and serological examinations.

and the CG solution was stored at −20° C until tested. Serum was obtained from all patients at the time of arthrocentesis. Sera, SF, and CG were coded and studied without knowledge of the diagnosis. The amounts of SF and CG solution were recorded for later quantitative calculations.

Serological Tests

Paired samples of sera, SF and CG were studied after heating to +37° C using indirect immunofluorescence technique. Washed normal leukocytes and cryostate sections of human thyroid tissues served as nuclear substrates as described earlier (34). Sera were screened at dilution 1/16, SF and CG undiluted, for the occurrence of GS-ANF and ON-ANF employing fluorescein isothiocyanate-labelled rabbit IgG specific for human γ, μ and α chains and the β1c-component of human C3 (Dakopatts, Copenhagen). Positive samples were titrated using PBS as diluent. The specificity of the conjugates was checked by crossed immunoelectrophoresis and direct immunofluorescence on monoclonal bone marrow specimens, and the working dilutions were chosen after determination of the plateau and point titres (34).

Heat-inactivated specimens (56° C, 30 minutes) of sera, SF and CG were investigated for rheumatoid factors by the latex fixation slide test (Hyland, Brussels) and by sensitized sheep cell agglutination test. Sera and SF containing titres of ≧ 32 by the latex fixation test and/or ≧ 20 by the sensitized sheep cell agglutination test were considered sero-positive. CG were regarded as positive if latex fixation was demonstrated using undiluted CG solutions.

Microscopy

All results were read in a Leitz Orthoplan epi-illumination fluorescence microscope equipped with interference and glass filters for detection of fluorescein isothiocyanate as described in detail elsewhere (32).

Protein Determinations

Total protein concentrations of CG were measured by the *Lowry* method as modified by *Lous et al.* (20). IgG dissolved in PBS served as protein standard.

IgG, IgA and IgM concentrations were determined by quantitative immunoelectrophoresis (29). CG were heat-dissolved just before applying the samples into the antigen wells, and the electrophoresis was run at room temperature.

Complement C1q, C4 and C3 were demonstrated by double immunodiffusion in 1 per cent agarose in PBS. Fibrinogen antigenic material was shown in the same way.

All antisera were obtained from Dakopatts, Copenhagen. They all showed monospecific reactions in crossed immunoelectrophoresis using whole human serum and/or plasma as antigens.

Statistical Methods

The X^2 test, the Wilcoxon rank sum test and the Wilcoxon test for pair differences (8). Significance level 5 per cent.

RESULTS

CG could be detected in all SF specimens from both groups of patients. Sufficient amounts for study were obtained from 42 of the 85 RA SF and from 18 of the 24 OA SF. 31 of the 42 RA patients showed positive reactions for rheumatoid factors in serum, SF or both. In all CG specimens, irreversibly insoluble material was present and hence, inaccessible for further study.

As shown in Table 2 most CG from RA patients were of the mixed type containing IgG + IgA or IgG + IgA + IgM. In contrast, more than half of the CG from OA patients were devoid of detectable Ig. The number of Ig-containing CG in the two groups was significantly different ($X^2 = 6.77$; $p < 0.01$).

Fibrinogen antigens were frequently demonstrated in both RA and OA CG (Table

TABLE 2 *Immunoglobulin Constituents* Detected in Synovial Fluid Cryoglobulins from 42 Rheumatoid Arthritis Patients and 18 Osteo-arthrosis Patients, (No.)*

	IgG-IgA-IgM	IgG-IgA	IgG	IgA	Ig not detected	Total
Rheum. arthritis	12	17	3	1	9	42
Osteo-arthrosis	2	5	1	0	10	18

* Studied by immunoelectrophoresis a. m. *Weeke* (29).

17*

(35) and the presence of Ig-containing inclusions in the leukocytes (5, 39).

In recent years, increasing evidence has been presented that cryoglobulins (CG) isolated from human sera represent cold-insoluble immune complexes (3, 18, 28). The most direct proof stems from the study of CG in animals with experimental serum sickness, where the isolated CG was greatly enriched with the immunizing antigen and specific antibody (12). Recently, CG containing immunoglobulins and deoxyribonucleic acid were demonstrated in a large percentage of SF from RA patients (7, 21), and antinuclear factors (ANF) and rheumatoid factors were found to participate in the cryoprecipitable material (7, 21, 31, 39). A considerable portion of the complement-fixing activity in SF was cryoprecipitable (27). It is thus conceivable that SF CG in RA may play a role in the chronic inflammatory processes by induction of local type III-reactions (10, 30, 37).

In an attempt to evaluate the possible pathogenetic significance of granulocyte-specific antinuclear factors (GS-ANF), so commonly found in RA sera and SF (9, 32), we have investigated CG from rheumatoid SF for occurrence of GS-ANF. Another aim of this study was to elucidate whether these proteins really represent immune complexes or mere cryofibrinogens containing trapped immunoglobulins from the SF. We have therefore done approximated quantitations of IgG, IgA and IgM as well as total protein content of the CG in RA and osteo-arthrosis (OA) patients. Fibrinogen-antigenic material, C1q, C4 and C3 has been looked for by double immunodiffusion.

The data obtained are compatible with the notion that SF CG in RA, at least in part, represent immune complexes containing GS-ANF and sometimes organ-nonspecific antinuclear factors (ON-ANF), both of which may be significantly enriched in the CG compared to the corresponding SF.

MATERIALS AND METHODS

77 patients fulfilling the criteria of the American Rheumatism Association for classical and definite RA (26) and 24 patients fulfilling generally accepted criteria for osteo-arthrosis including normal erythrocyte sedimentation rate and serum electrophoresis and typical clinical and radiological features, all admitted to the Department of Rheumatology, Frederiksberg Hospital, Copenhagen, were selected for the study solely on the basis of a presence of sufficient amounts of available SF. The age and sex distribution of the patients is shown in Table I.

SF was aspirated under sterile conditions into tubes containing EDTA in a final concentration of about 0.01 mMol/ml. Cells were removed by centrifugation at 400 × g for ten minutes, and the supernatant was incubated with testicular hyaluronidase (Penetrase, LEO, Copenhagen) 150 IU/ml SF for thirty minutes at +37° C. The SF was incubated at +4° C for 24 hours, and cryoprecipitable material was pelleted at 1000 × g for ten minutes. The supernatant SF was stored at −20° C. The CG was then resuspended and washed four times in ice cold phosphate buffered saline pH 7.2 (PBS) in an attempt to remove nonspecifically trapped proteins from the precipitate, and the CG was redissolved in a small volume of PBS at +37° C. Insoluble material, equally present in RA and OA specimens, was removed by centrifugation

TABLE I. *Age and Sex Distribution of All Patients*

	Cryoglobulin isolated*	No.	Mean	Range	Men/women
Rheum. arthritis	+	42	64.5	32–83	12/30
	—	35	66.3	43–87	9/26
Osteo-arthrosis	+	18	75.4	37–88	8/10
	—	6	74 8	64–82	0/ 6

* Sufficient amount of cryoglobulin in the synovial fluid to allow isolation, washing, measurement of protein, IgG, IgA and IgM and serological examinations.

and the CG solution was stored at −20° C until tested. Serum was obtained from all patients at the time of arthrocentesis. Sera, SF, and CG were coded and studied without knowledge of the diagnosis. The amounts of SF and CG solution were recorded for later quantitative calculations.

Serological Tests

Paired samples of sera, SF and CG were studied after heating to +37° C using indirect immunofluorescence technique. Washed normal leukocytes and cryostate sections of human thyroid tissues served as nuclear substrates as described earlier (34). Sera were screened at dilution 1/16, SF and CG undiluted, for the occurrence of GS-ANF and ON-ANF employing fluorescein isothiocyanate-labelled rabbit IgG specific for human γ, μ and α chains and the β1c-component of human C3 (Dakopatts, Copenhagen). Positive samples were titrated using PBS as diluent. The specificity of the conjugates was checked by crossed immunoelectrophoresis and direct immunofluorescence on monoclonal bone marrow specimens, and the working dilutions were chosen after determination of the plateau and point titres (34).

Heat-inactivated specimens (56° C, 30 minutes) of sera, SF and CG were investigated for rheumatoid factors by the latex fixation slide test (Hyland, Brussels) and by sensitized sheep cell agglutination test. Sera and SF containing titres of $\geqq$ 32 by the latex fixation test and/or $\geqq$ 20 by the sensitized sheep cell agglutination test were considered sero-positive. CG were regarded as positive if latex fixation was demonstrated using undiluted CG solutions.

Microscopy

All results were read in a Leitz Orthoplan epi-illumination fluorescence microscope equipped with interference and glass filters for detection of fluorescein isothiocyanate as described in detail elsewhere (32).

Protein Determinations

Total protein concentrations of CG were measured by the *Lowry* method as modified by *Lous et al.* (20). IgG dissolved in PBS served as protein standard.

IgG, IgA and IgM concentrations were determined by quantitative immunoelectrophoresis (29). CG were heat-dissolved just before applying the samples into the antigen wells, and the electrophoresis was run at room temperature.

Complement C1q, C4 and C3 were demonstrated by double immunodiffusion in 1 per cent agarose in PBS. Fibrinogen antigenic material was shown in the same way.

All antisera were obtained from Dakopatts, Copenhagen. They all showed monospecific reactions in crossed immunoelectrophoresis using whole human serum and/or plasma as antigens.

Statistical Methods

The X^2 test, the Wilcoxon rank sum test and the Wilcoxon test for pair differences (8). Significance level 5 per cent.

RESULTS

CG could be detected in all SF specimens from both groups of patients. Sufficient amounts for study were obtained from 42 of the 85 RA SF and from 18 of the 24 OA SF. 31 of the 42 RA patients showed positive reactions for rheumatoid factors in serum, SF or both. In all CG specimens, irreversibly insoluble material was present and hence, inaccessible for further study.

As shown in Table 2 most CG from RA patients were of the mixed type containing IgG + IgA or IgG + IgA + IgM. In contrast, more than half of the CG from OA patients were devoid of detectable Ig. The number of Ig-containing CG in the two groups was significantly different ($X^2 = 6.77$; $p < 0.01$).

Fibrinogen antigens were frequently demonstrated in both RA and OA CG (Table

TABLE 2 *Immunoglobulin Constituents* Detected in Synovial Fluid Cryoglobulins from 42 Rheumatoid Arthritis Patients and 18 Osteo-arthrosis Patients, (No.)*

	IgG-IgA-IgM	IgG-IgA	IgG	IgA	Ig not detected	Total
Rheum. arthritis	12	17	3	1	9	42
Osteo-arthrosis	2	5	1	0	10	18

* Studied by immunoelectrophoresis a.m. *Weeke* (29).

TABLE 3. *Double Immunodiffusion Studies of Cryoglobulins Isolated from Synovial Fluid, (No.)*

	C1q	C4	C3	Fibrinogen
Rheum. arthritis	4/38	0/41	2/41	21/36
Osteo-arthrosis	0/13	0/16	1/16	7/14

TABLE 4. *Quantitation of Total Protein, IgG, IgA and IgM in Cryoglobulins from Synovial Fluids of Rheumatoid Arthritis and Osteo-arthrosis Patients (mg/100 ml Synovial Fluid)**

	Rheumatoid arthritis			Osteo-arthrosis			p§
	Mean	Range	Found in (%)	Mean	Range	Found in (%)	
Total protein	19.6	0.9–51.8	42/42 (100)	7.0	0.7–24.6	18/18 (100)	<0.05
IgG	1.68	0 –12.02	32/42 (76)	0.36	0 – 1.89	8/18 (44)	<0.01
IgA	0.17	0 – 1.89	30/42 (71)	0.04	0 – 0.27	7/18 (39)	<0.01
IgM	0.05	0 – 0.48	12/42 (29)	0.01	0 – 0.04	2/18 (11)	n.s.

* All quantitations were done on the concentrated cryoglobulin solutions. The values shown in the table are corrected to show the concentration in the synovial fluid it was derived from by the calculation: $x = y \frac{\text{CG solution volume}}{\text{SF volume}}$; y = measured value.

§ Wilcoxon's rank sum test.

TABLE 5. *The Synovial Fluid Cryoglobulin Content of IgG, IgA and IgM Related to the Amount of Total Protein in the Cryoglobulin (%)*

	Rheumatoid arthritis		Osteo-arthrosis		p*
	Mean	Range	Mean	Range	
IgG	12.96	0–46.30	2.68	0–20.9	<0.01
IgA	1.76	0– 7.30	0.34	0– 2.69	<0.01
IgM	0.47	0– 5.56	0.03	0– 0.26	n.s

* Wilcoxon's rank sum test.

3) while complement components were rare in both groups.

If measured quantitatively the concentration of total protein, IgG and IgA could be higher in RA than in OA CG (Table 4). Moreover, IgG and IgA constituted a higher percentage of the total protein concentration in RA CG (Table 5). However, since the levels of IgG, IgA and IgM in RA SF were significantly higher than in OA SF (Table 6) we felt it more correct to relate the amount of these three Ig classes in the CG to that of the SF from which it was derived to outrule simple trapping of these proteins in a precipitate of cryofibrinogen (Table 7). A definite trend towards higher relative values was still visible in the RA specimens, though statistically not significantly different from OA specimens.

Altogether 26 rheumatoid CG contained GS-ANF of the IgG and/or IgM classes (62 per cent). As depicted in Table 8, about 50 per cent of the CG showed IgG GS-ANF activity. IgM GS-ANF occurred equally frequent. 7 CG contained IgG GS-ANF only, 9 IgM GS-ANF only and 10 both IgG and IgM GS-ANF. ON-ANF of the IgG, IgA and IgM classes were found in 13 CG (30 per cent). About one fifth had IgG ON-ANF activity and one fifth IgM ON-ANF activity

TABLE 6. *Quantilation of IgG, IgA and IgM in Synovial Fluids of Patients from Which Cryoglobulins Were Isolated (mg/100 ml)*

	Rheumatoid arthritis		Osteo-arthrosis		p*
	Mean	Range	Mean	Range	
IgG	970	340–1880	423	260–710	<0.01
IgA	104	27– 354	52	20–200	<0.01
IgM	45	9– 161	14	6– 30	<0 01

* Wilcoxon's rank sum test.

TABLE 7. *The Synovial Fluid Cryoglobulin Content of IgG, IgA and IgM Related to the Total Amount of IgG, IgA and IgM in the Corresponding Synovial Fluid (%)*

	Rheumatoid arthritis		Osteo-arthrosis		p*
	Mean	Range	Mean	Range	
IgG	0.16	0–0.79	0.08	0–0.40	n.s. (<0.1)
IgA	0.19	0–1.89	0.10	0–0.70	n.s. (<0.1)
IgM	0.11	0–1.00	0.02	0–0.17	n.s.

* Wilcoxon's rank sum test.

TABLE 8. *Comparison between GS-ANF, ON-ANF and Rheumatoid Factors in Cryoglobulins and Corresponding Synovial Fluids from Rheumatoid Arthritis Patients*

	IgG GS-ANF	IgA GS-ANF	IgM GS-ANF	IgG ON-ANF	IgA ON-ANF	IgM ON-ANF	Rheum. fact.
G pos./SF pos.	15 } 47%*	0	17 } 53%*	7 } 19%*	0	6 } 17%*	8 } 24%*
G pos./SF neg.	2	0	2	1	1	1	2
G neg./SF pos	13	8	11	9	10	13	20
G neg./SF neg.	6	27	6	25	31	22	12

* Percentage of positive cryoglobulins.

(Table 8). 5 CG contained IgG ON-ANF only, 4 IgM ON-ANF only and 3 both IgG and IgM ON-ANF. Rheumatoid factors were demonstrated in 24 per cent of the RA CG.

GS-ANF and ON-ANF were generally found in CG derived from SF containing the same Ig class and specificity of ANF, but in a few cases such antibodies could only be detected in the CG. The same was true of the antiglobulins which in only two cases were demonstrated exclusively in the CG.

In patients exhibiting positive tests for ANF in both CG and SF, an attempt was made to elucidate whether or not enrichment of these specific antibodies had occurred in the CG (Table 9). Significantly higher titres per gramme of IgG were found for both IgG GS-ANF and IgG ON-ANF in the CG.

IgG or IgM ANF were not detected in the CG in any case of OA, while one case showed presence of IgA ON-ANF in the CG as well as in the SF. There was no enrichment of this antibody in the CG. IgG ON-ANF was found in a single SF, IgM GS-ANF in 8 and IgM ON-ANF in 5 SF.

DISCUSSION

The demonstration of reduced levels of haemolytic complement in SF of RA patients

TABLE 9. Calculated Values of IgG GS-ANF and ON-ANF per Gramme of Cryoglobulin IgG and Synovial Fluid IgG in RA Patients

Sample pair no.	Cryoglobulin IgG GS-ANF titer/g IgG	Synovial fluid IgG GS-ANF titer/g IgG	
15	166.7	9.2	
21	238.1	92.8	
29	185.2	42.1	
36	96.2	19.0	
42	75.8	5.1	
47	41.7	14.3	
49	2353.0	269.5	
55	350.9	124.3	$p<0.05$*
79	400.0	152.4	
88	15.9	36.0	
117	125.0	502.0	
134	4000 0	517.2	
161	655.7	2301.1	
146	1000.0	79.0	
151	1000.0	1.6	
	IgG ON-ANF titer/g IgG	IgG ON-ANF titer/g IgG	
29	46.3	10.5	
43	56.9	23.7	
49	588.2	67.4	$p=0.05$*
55	87.7	15.5	
126	61.0	3.9	
134	500.0	64.6	

* *Wilcoxon's test for pair differences.*

(15, 24), in contrast to increased or normal levels in serum, indicate that complement-consuming immune reactions take place in the synovium.

Townes & Marcus (27) clearly showed complement-fixing activity in RA SF, such activity being more pronounced in sero-positive than in sero-negative cases. Cryoprecipitable material from these fluids was responsible for the major part of the complement fixation. Further studies indicated that the most efficient complement fixation was exhibited by high molecular weight complexes composed of IgG and IgM or IgG alone (21). Non-rheumatoid CG were generally unable to fix complement, rarely contained IgG but often fibrinogen. The RA CG showed presence of one or more Ig classes, complement components, DNA and fibrinogen (7, 21, 31, 39). Some of the antibodies had ANF and antiglobulin activity.

ANF and antiglobulins appeared to be enriched in some CG compared to SF (7).

The present study has shown cold-precipitable material both in sero-positive and sero-negative rheumatoid and non-rheumatoid SF, but a considerable part of the CG became irreversibly insoluble upon cooling, probably representing cross-linked cryofibrinogen (11). Fibrinogen antigens could be identified in most CG solutions even after removal of the insoluble material.

The total amounts of protein, IgG and IgA were higher in RA than in OA CG. In the rheumatoid CG, the measured quantity of each Ig class possibly represent minimum values since heat dissolution of the precipitates carried out just before the electrophoretic run cannot be expected to cause complete dissociation of immune complexes (17, 18, 28). On the other hand, we preferred to measure the different Ig classes by quantitative immunoelectrophoresis rather than by radial immunodiffusion, since it was anticipated from immunoelectrophoretic studies on CG done by other investigators (17, 18, 28) that the dissociative forces exerted on the CG constituents during the electrophoresis partly can overcome the weak associative forces caused by the immune reaction. Very loosely bound immune reactants were described by *Marcus & Townes* (21) who had to desist from washing SF CG because this caused loss of complement components otherwise selectively attached to RA CG. Our washing procedure, therefore, most probably explains why complement proteins were rarely detected in the CG

IgG and IgA constituted a larger percentage of the CG protein in RA than in OA. However, even simple trapping of Ig in a network of cryofibrinogen would lead to higher relative amounts in RA CG, as the level of all three Ig classes was significantly higher in the corresponding SF. Therefore, we related the amount of each Ig constituent to the level in the matched SF. Though a trend towards higher relative values was seen

in RA compared to OA specimens, significant differences were not found.

In a previous study we showed IgG ANF in about 82 per cent of RA SF (32). The high prevalence was primarily due to the frequently occurring GS-ANF which were demonstrated in about 69 per cent of all the specimens. The present study indicate that GS-ANF are very common participants in rheumatoid SF CG, and further confirms the presence of ON-ANF and antiglobulins in some CG (7, 21, 39). In contrast to the latter study, we only found few cases where ANF or antiglobulins were demonstrable in the CG and not in the SF. However, when comparison between CG and SF as to the level of IgG ANF per gramme of IgG was made, significantly higher titres were found in the CG.

Whether these data reflect real enrichment of specific antibodies in the CG is debatable, since antigenic inhibitors of ANF (2) and antiglobulins (13) may be found in some RA SF and hence interfere with their demonstration. On the other hand, the actual content of ANF and antiglobulins in the CG is probably higher than that recorded after heat dissolution only. This is deduced from preliminary observations on six rheumatoid SF CG which on heat dissolution only showed negative reactions for immunoconglutinin, another auto-antibody likely to participate in complement-binding immune complexes (19). After pepsin digestion, four CG revealed clearly positive immunoconglutinin activity, while similarly treated OA CG were negative both before and after pepsin treatment (33).

Our data conform to the conception that rheumatoid SF CG partly represent cold-insoluble immune complexes built up by nuclear antigens, ANF, antiglobulins, complement and immunoconglutinins. If enrichment of the CG with GS-ANF and ON-ANF can be substantiated, this will firmly support the hypothesis, and further establish the potentiality of ANF to participate in the pathogenesis of the articular inflammation.

We express our gratitude to professor *Viggo Faber* for helpful advice and criticism. The skilful technical assistance of Mrs. *Tove Dietz*, Mrs. *Merete Skånild*, Mrs. *Hanne Sørensen* and Miss *Åse Stricker-Nielsen* is gratefully acknowledged.

This study was supported by grants from the *National Danish Association Against Rheumatic Diseases*, the *Danish Medical Research Council* grant no. 512-3212, the *Hindsgaul Foundation* and the *NOVO Foundation*.

REFERENCES

1. *Agnello, V., Winchester, R. J. & Kunkel, H. G.:* Precipitin reactions of the C1q component of complement with aggregated IgG and immune complexes in gel diffusion. Immunology *19:* 909–919, 1970.
2. *Barnett, E. V.:* Detection of nuclear antigens (DNA) in normal and pathologic human fluids by quantitative complement fixation. Arthritis Rheum. *11:* 407–417, 1968.
3. *Barnett, E. V., Bluestone, R., Cracchiolo, A., Goldberg, L. S., Kantor, G. & McIntosh, R. M.:* Cryoglobulinemia and disease. Ann. Intern. Med. *73:* 95–107, 1970.
4. *Brandt, K. D., Cathcart, E. S. & Cohen, A. S.:* Studies of immune deposits in synovial membranes and corresponding synovial fluids. J. Lab. Clin. Med. *72:* 631–647, 1968.
5. *Britton, M. C. & Schur, P. H.:* The complement system in rheumatoid synovitis. II. Intracytoplasmic inclusions of immunoglobulins and complement. Arthritis Rheum. *14:* 87–95, 1971.
6. *Cracchiolo, A. & Goldberg, L. S.:* Elution of antiglobulins and antinuclear antibody from rheumatoid synovial membrane. Ann. rheum. Dis. *31:* 186–191, 1972.
7. *Cracchiolo, A., Goldberg, L. S., Barnett, E. V. & Bluestone, R.:* Studies of cryoprecipitates from synovial fluid of rheumatoid patients. Immunology *20:* 1067–1077, 1971.
8. *Diem, K.:* Scientific Tables. Documenta Geigy, J. R. Geigy, Basel 1962, pp 36, 124, 128.
9. *Elling, P., Graudal, H. & Faber, V.:* Granulocyte-specific antinuclear factors in serum and synovial fluid in rheumatoid arthritis. Ann. rheum. Dis. *27:* 225–233, 1968.
10. *Gell, P. G. H. & Coombs, R. R. A.:* The classification of allergic reactions responsible for clinical hypersensitivity and disease. In: Gell, P. G. H. & Coombs, R. R. A. (Eds.): Clinical Aspects of Immunology, 2. ed. Blackwell Scientific Publications Ltd., Oxford 1968, p. 575–596.
11. *Glueck, H. I. & Herrmann, L. G.:* Cold-precipitable fibrinogen, "cryofibrinogen". Arch. Int. Med. *113:* 748–757, 1964.

12. *Griswold, W. R.:* Selective concentration of specific antibody in serum cryoprecipitates. Int. Arch. Allergy *47:* 242–248, 1974.
13. *Hannestad, K.:* Presence of aggregated γG-globulin in certain rheumatoid synovial effusions. Clin. exp. Immunol. *2:* 511–529, 1967.
14. *Hannestad, K.:* Rheumatoid factors reacting with autologous native γG-globulin and joint fluid γG aggregates. Clin. exp. Immunol. *3:* 671–690, 1968.
15. *Hedberg, H.:* The depressed synovial complement activity in adult and juvenile rheumatoid arthritis. Acta Rheum. Scand. *10:* 109–127, 1964.
16. *Hollander, J. L., McCarthy, D. J., Astorga, G. & Castro-Murillo, E.:* Studies on the pathogenesis of rheumatoid joint inflammation. Ann. Intern. Med. *62:* 271–280, 1965.
17. *Klein, F., van Rood, J. J., van Furth, R. & Randema, H.:* IgM-IgG cryoglobulinaemia with IgM paraprotein component. Clin. exp. Immunol. *3:* 703–716, 1968.
18. *Klein, M., Danon, F., Brouet, J. C., Signoret, Y. & Seligmann, M.:* Étude immunochimique de 130 cryoglobulines humaines. Rév. europ. études clin. biol. *17:* 948–957, 1972.
19. *Lachmann, P. J.:* Conglutinin and immunoconglutinins. Advan. Immunol. *6:* 479–527, 1967.
20. *Lous, P., Plum, C. M. & Schou, M.:* Kolorimetrisk bestemmelse af totalprotein og globulin i spinalvæske. Nord. Med. *55:* 693–695, 1956.
21. *Marcus, R. L. & Townes, A. S.:* The occurrence of cryoproteins in synovial fluid: the association of a complement-fixing activity in rheumatoid synovial fluid with cold-precipitable protein. J. Clin. Invest. *50:* 282–293, 1971.
22. *Munthe, E. & Natvig, J. B.:* γ globulin complexes and anti-γ globulin antibodies in rheumatoid tissue and tissue eluates. In: Bonomo, L. & Turk, J. L. (Eds.) Proceedings of the International Symposium on Immune Complex Diseases. Carlo Erba Foundation, Milan 1970, p. 86–87.
23. *Munthe, E. & Natvig, J. B.:* Characterization of IgG complexes in eluates from rheumatoid tissue. Clin. exp. Immunol. *8:* 249–262, 1971.
24. *Pekin, T. J. jr. & Zvaifler, N. J.:* Hemolytic complement in synovial fluid. J. Clin. Invest. *43:* 1372–1382, 1964.
25. *Rodman, W. S., Williams, R. C. jr., Bilka, P. J. & Müller-Eberhard, H. J.:* Immunofluorescent localization of the third and fourth component of complement in synovial tissue from patients with rheumatoid arthritis J. Lab. Clin. Med. *69:* 141–150, 1967.
26. *Ropes, M. W., Bennett, G. A., Cobb, S., Jacox, R. & Jessar, R. A.:* 1958 revision of diagnostic criteria for rheumatoid arthritis. Arthritis Rheum. *2:* 16–18, 1958.
27. *Townes, A. S. & Marcus, R. L.:* Complement-fixing activity of rheumatoid synovial fluid. Ann. rheum. Dis. *31:* 393–401, 1972.
28. *Wager, O. & Räsänen, J. A.:* Mixed cryoglobulinemia in relation to autoimmune aberrations. In: Bonomo, L. & Turk, J. L. (Eds.) Proceedings of the International Symposium on Immune Complex Diseases. Carlo Erba Foundation, Milan 1970, p. 140–148
29. *Weeke, B.:* Quantitative estimation of human immunoglobulins following carbamylation by electrophoresis in antibody containing agarose gel. Scand. J. Clin. Lab. Invest. *22:* 107–111, 1968.
30. *Weismann, G.:* Lysosomes and joint disease. Arthritis Rheum. *9:* 834–840, 1966.
31. *Wiik, A., Bach-Andersen, R., Friis, J. & Jensen, E. M.:* Immunochemical and serological investigations of cryoprecipitates from synovial fluids. Scand. J. Rheumatol. *2:* 9, 1973.
32. *Wiik, A., Jensen, E & Friis, J.:* Granulocyte-specific antinuclear factors in synovial fluids and sera from patients with rheumatoid arthritis. Ann. rheum. Dis.*33:* 515–522, 1974.
33. *Wiik, A. & Mellbye, O* Unpublished work.
34. *Wiik, A. & Munthe, E:* Restrictions among heavy and light chain determinants of granulocyte-specific antinuclear factors. Immunology *23: 53–60, 1972*
35. *Winchester, R J., Agnello, V. & Kunkel, H. G.:* γ globulin complexes in synovial fluids of patients with rheumatoid arthritis Partial characterization and relationship to lowered complement levels Clin. exp. Immunol. *6:* 689–706, 1970.
36 *Winchester, R. J., Kunkel, H. G. & Agnello, V.:* Occurrence of γ globulin complexes in serum and joint fluid of rheumatoid arthritis patients: Use of monoclonal rheumatoid factors as reagents for their demonstration. J. exp. Med. *1934*. 286–295, 1971.
37. *Zvaifler, N. J.:* Further speculation on the pathogenesis of joint inflammation in rheumatoid arthritis. Arthritis Rheum. *13:* 895–901, 1970.
38 *Zvaifler, N. J.:* Rheumatoid synovitis. An extravascular immune complex disease. Arthritis Rheum *17:* 297–305, 1974.
39. *Zvaifler, N. J.:* The immunopathology of joint inflammation in rheumatoid arthritis Advan Immunol. *16.* 265–336, 1973.

Acta path. microbiol. scand. Sect. C, *83:* 273–279, 1975

URINARY EXCRETION OF GRANULOCYTE-SPECIFIC ANTINUCLEAR FACTORS IN RHEUMATOID ARTHRITIS

Predominance in Neutropenic Cases Showing High Titres of Complement Fixation

ALLAN WIIK, KIRSTEN HENRIKSEN and VIGGO FABER

Immunological Laboratory, University Clinic for Infectious Diseases, Blegdamshospitalet, Copenhagen, Denmark

Wiik, A., Henriksen, K. & Faber, V. Urinary excretion of granulocyte-specific antinuclear factors in rheumatoid arthritis. Predominance in neutropenic cases showing high titres of complement fixation. Acta path. microbiol. scand. Sect. C, *83:* 273–279, 1975.

Sera and 24-hour urine samples were obtained from 36 patients with rheumatoid arthritis. On the basis of precedent serum studies the patients were divided into two groups, group A consisting of 20 patients showing varying titres of IgG granulocyte-specific antinuclear factors, almost exactly corresponding to the titres in the 16 group B patients, but differing as to the complement-fixing properties of the specific antibodies which were restricted to group A only. In five group A patients and one group B patient, an increased excretion of total protein in the urine and an abnormal agarose electrophoresis pattern of the concentrated proteins suggested kidney damage which could not be accounted for by previous or recent medication or clinically explicable nephropathy. IgG granulocyte-specific antinuclear factors were present in urines from 12 group A patients and 3 group B patients. A direct relation between high titres of complement-fixing granulocyte-specific antinuclear factors and presence of the specific antibodies in the urine was demonstrated. In one group A patient with a classical Felty's syndrome, a renal biopsy disclosed immune complex deposition, though clinical and laboratory signs of glomerulopathy were absent. It is suggested that complement-fixing granulocyte-specific antinuclear factors may occupy a position comparable to that of complement-fixing anti-DNA in systemic lupus erythematosus as regards the induction of immune complex deposition and eventually glomerulitis.

Key words. Rheumatoid arthritis; antinuclear factors; urinary excretion.

A. Wiik, Immunological Laboratory, University Clinic for Infectious Diseases, Blegdamshospitalet, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark.

Received 5.ii.75 Accepted 28.ii.75

Organ-nonspecific antinuclear factors (ON-ANF) with pronounced complement-fixing (CF) properties may be of pathogenetic significance for the induction of immune complex nephritis in systemic lupus erythematosus (SLE) (23, 29). This is supported by the demonstration of nuclear antigens and ON-ANF in the immune complex deposits (16, 17). In cases of active lupus nephritis, ON-ANF often appear in the urine (4); hence, discovery of such antibodies in the

urine could, by way of deduction, indicate preclinical renal involvement.

It is well-established that demonstration of 'granulocyte-specific antinuclear factors (GS-ANF) is of practical value for the diagnosis of *rheumatoid arthritis (RA)* (7, 32). However, the pathogenetic significance of these antibodies remains to be substantiated. It has been suggested that GS-ANF with pronounced CF properties may play a role in the induction of the neutropenia found in a minority of RA patients (34). This study aims at elucidating whether CF GS-ANF may influence the renal excretion of GS-ANF and whether this may signify rheumatoid glomerular disease.

MATERIALS AND METHODS

Sera were obtained from 20 patients who on routine analysis were found to have CF GS-ANF in serum (group A); they were all suffering from classical or definite RA (24). Twelve of these patients showed neutropenia (<2000 neutrophils/µl). By way of comparison, sera were selected from sixteen RA patients without neutropenia who had similar titres of IgG GS-ANF, but no CF GS-ANF (group B).

Twenty-four-hour samples of urine from these patients were collected into sodium azide, final concentration about 0.015 M/l. Urine was concentrated to about 25 g/l total protein by low pressure ultrafiltration at 4° C, using Visking tube 23/32 (8), and the concentrated samples were dialysed in the cold against phosphate buffered saline pH 7.2 containing 0.015 M sodium azide/l (PBS). Sera and specimens of concentrated urine were stored at -20° C until tested. *Protein concentrations* in unconcentrated urine were determined according to *Savory et al* (26), while concentrated specimens were studied by a standard biuret method. Electrophoresis of the urinary proteins was performed in 1 per cent agarose (w/v) in 0.075 M veronal buffer pH 8.6 for 60 minutes at 20 V/cm (14). Classification of the electrophoresis patterns was according to *Laurell* (19).

Serial dilutions of sera and urine concentrates in PBS were investigated for IgG and CF GS-ANF and ON-ANF by indirect immunofluorescence technique as described previously (33). Fluorescein isothiocyanate-labelled rabbit IgG specific for human γ chains and the β1c-component of C3 was purchased from Dakopatts, Copenhagen. Before use, the conjugates were tested for specificity, F/P-ratio and working dilution (34). All results were read in a Leitz Orthoplan fluorescence microscope equipped with incident light illumination, using interference filters as described before (32).

Renal biopsy material from one patient was kindly studied by *Svend Larsen*, Kommunehospitalet, Copenhagen, by direct immunofluorescence technique.

The statistical significance of the data obtained was evaluated by the χ^2-test and the Wilcoxon rank sum test (6). Significance level 5 per cent.

RESULTS

The urinary excretion of protein is shown in Table 1. The upper normal limit of protein excretion (150 mg/24 hours) is based on data from normal control persons (14). According to these criteria, however, about 40 per cent of the patients in both groups had proteinuria; the more severe proteinuria in group A patients is illustrated by the fact that six patients in group A and only two in group B excreted more than 300 mg/24 hours. At the time of study, a nephrotic syndrome was found in only one patient in group A. She suffered from a classical Felty's syndrome, excreted about 9–10 g of protein/24 hours and serum creatinine was slightly raised (around 2 mg per cent). No clinical signs of amyloidosis were present and biopsies from the rectal mucosa contained no amyloid. Renal biopsy was not performed because of the very advanced and clinically poor condition of the patient. None of the patients showed renal insufficiency.

The agarose electrophoresis pattern of urine proteins was normal in all patients excreting <150 mg of protein/24 hours, while seven patients in group A and three in group B excreting >150 mg/24 hours showed an abnormal pattern. In two group A patients and two group B patients, these abnormalities may be explained on the basis of clinical data. One group A patient with Felty's syndrome had an acute attack of pyelonephritis at the time of study, and another group A patient with RA had been suffering from chronic pyelonephritis for years, the serum creatinine level remaining steady around 2 mg per cent. Both showed a tubular electrophoresis pattern. At the time of study, one group B patient was receiving gold

TABLE 1. *Urinary Protein Excretion in Two Groups of RA Patients. Group A: Patients with GS-ANF with CF Properties and Group B: Patients with GS-ANF without CF Properties in Serum*

Group	No. of patients	Protein excretion mgs/24 hours (range)	No. of patients	Electrophoresis pattern*	No. of patients
A	20	<150 (59–129)	12	Normal	12
		>150 (213–9514)	8	Normal Glomerular Tubular	1 4 3
B	16	<150 (67–141)	10	Normal	10
		>150 (186–952)	6	Normal Glomerular Tubular	3 3 0

* Electrophoresis pattern classified according to *Laurell* (19).

TABLE 2. *Urinary Excretion of IgG GS-ANF*

Group	No. of patients	Serum titre of IgG GS-ANF, median and range	IgG GS-ANF present in urine (no.)
A	20	512 16–64,000	12
B	16	512 16–32,000	3

treatment, which may explain the glomerular pattern (27), while one patient who had abused phenacetin for years showed a somewhat similar pattern. Serum creatinine was normal in both. In the remaining five group A patients and one group B patient kidney involvement, manifest in an abnormal excretion of protein and urine electrophoresis pattern could not be explained.

IgG GS-ANF were found in urine from about 60 per cent of the patients in group A, as compared with about 19 per cent in group B, $\chi^2 = 13.16$; $p<0.001$ (6) (Table 2). A direct relation between high titres of CF GS-ANF in serum and appearance of IgG GS-ANF in the urine could be seen, $p<0.01$ (6) (Fig. 1). In contrast, no association to the IgG GS-ANF titres in serum could be seen, $p<0.1$ (6) (Fig. 2).

A percutaneous renal biopsy was obtained from one patient suffering from a classical Felty's syndrome who lacked overt signs of glomerulopathy, and in whom protein excretion and serum creatinine as well as urine sediment were normal. The serum titre of CF GS-ANF and IgG GS-ANF was 512 and 4096, respectively, and she excreted GS-ANF in the urine. Immunofluorescence staining showed widespread, finely granular deposits of IgM and complement-C3 along the glomerular basement membranes and in the mesangium. In addition, C3 was concentrated in single segments of the glomeruli and along the Bowman's capsule, a characteristic feature in some cases of SLE and RA (18).

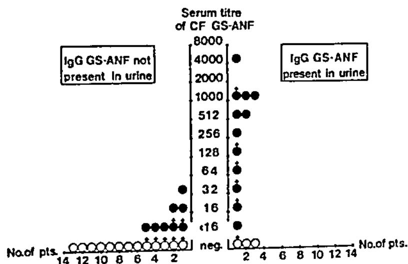


Fig. 1. Relation between serum titre of CF GS-ANF and appearance of IgG GS-ANF in the urine. All patients had IgG GS-ANF in serum, but some showed CF properties (●) and some did not (○). Patients who excreted >150 mg of protein in the urine per 24 hours (+).

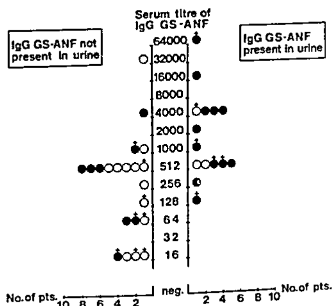


Fig. 2. Relation between serum titre of IgG GS-ANF and appearance of such antibodies in the urine. ● Positive CF. ○ Negative CF. + Protein excretion >150 mg/24 hours.

DISCUSSION

Gross similarities between SLE and certain forms of RA in which extra-articular manifestations such as Felty's syndrome were prominent have lead some authors to the conclusion, that Felty's syndrome merely is one form of SLE running a chronic course (5). On the other hand, a different histopathological picture and the fact that the renal and the central nervous system rarely are

involved in Felty's syndrome, may be forwarded as arguments against this notion (1, 25).

Differences in the serology are also seen. In sera from SLE patients, ON-ANF of the IgG class in high titres are commonly found (29, 31). In cases of active lupus nephritis, these antibodies show considerable CF properties and have been regarded to be of pathogenetic significance for the induction of the immune complex nephritis (23, 29). A presence of nuclear antigens and ANF in the glomerular lesions (16, 17) and in the urine (4) favours the hypothesis that circulating immune complexes containing nuclear antigens and ANF deposit in the glomeruli, bind and activate complement and initiate type III reactions (15) which cause the vascular damage (10). In this regard, the CF properties of the auto-antibodies may be more important than the specificity (29).

IgG ON-ANF in Felty's syndrome usually lack CF properties (3, 34). In contrast, the GS-ANF which nearly invariably are present in high titres in these patients, as a rule show CF abilities (34). The present study shows that leakage of GS-ANF into the urine is seen mainly in patients whose serum contains CF GS-ANF (60 per cent). This is especially true of patients with high titres of CF GS-ANF as seen in cases of neutropenic RA. GS-ANF appeared in the urine of all of the 9 patients in whom serum titres of CF GS-ANF were 64 or more, neutropenia being in evidence in all of these patients. A simple prerenal mechanism of the GS-ANF excretion seems unlikely since any association between serum titres of IgG GS-ANF and appearance of these antibodies in the urine was not found.

Several studies suggest that circulating immune complexes are common in RA (11, 22, 35). Though glomerulitis may be detected in a low percentage in renal biopsies from RA patients (2), clinical signs of non-amyloidotic glomerulopathy are rarely seen to develop spontaneously (28). The demonstration of IgM and complement deposits in the glomeruli of one of our Felty patients suggests immune complex deposition. Owing to the presence of thrombocytopenia, the biopsy could not be performed while the disease was at the height of disease activity and accordingly, the deposition may not be representative of Felty's syndrome during exacerbations.

A direct association between cryoglobulinaemia and glomerulonephritis exists (20, 21). It is therefore worth noting that cryoglobulins very recently were demonstrated in sera from Felty patients (30). The cryoglobulins were rich in ANF reacting predominantly with granulocyte nuclei and showed clear CF properties. We have confirmed this by the finding of high titres of CF GS-ANF in serum cryoglobulins from two Felty patients, and showing that CF high molecular weight IgG GS-ANF are seen in Felty sera fractionated on G-200 gel columns at neutral pH (31). CF GS-ANF were eluted in the 7S peak if the same sera were fractionated at acid pH indicating immune complex dissociation.

The available data conform to the conception that Felty's syndrome is a form of RA located at one extreme of the RA disease spectrum manifesting generalized vasculitis and hyperplasia of the reticulo-endothelial system (1, 25), both of which may be directly or indirectly caused by large amounts of circulating immune complexes (30, 31). The hypocomplementaemia observed in some patients (9, 13) may be a result of a relative imbalance between complement production and consumption, the neutropenia being the result of a relative imbalance between bone marrow release and removal of neutrophils continually attempting to minimize the level of circulating immune complexes by phagocytosis. In patients excreting GS-ANF in the urine the same basic immunological dysfunction may be reflected which leads to the formation and persistence of immune complexes in the blood, thus causing vasculitis whether or not neutropenia is present. In short, our data fit into this scheme, showing that GS-ANF are important elements in the immune complexes, probably contributing to the CF properties of the immune complexes

(30, 31) and thus to the hypocomplementaemia. Though immune complex deposition may remain clinically quiescent for years (12) it could effect the glomerular penetration of GS-ANF described in this study. Well-documented glomerulonephritis may develop in some RA patients, but further studies are needed before this question can be definitely answered.

We gratefully acknowledge the expert assistance of Dr. *Svend Larsen* and the help obtained from departments of clinical medicine and rheumatology in Copenhagen. *Mrs. Tove Dietz* is thanked for skilful technical assistance.

This study was supported by grants from the *National Danish Association Against Rheumatic Diseases*, the *Danish Medical Research Council*, grant no. 512-3212 and 512-3648 and the *Købmand i Odense Johann og Hanne Weimann Foundation*.

REFERENCES

1. *Barnes, C. G., Thurnbull, A. L. & Vernon-Roberts, B.:* Felty's syndrome. Ann. rheum. Dis. *30:* 359–374, 1971.
2. *Berman, L. B. & Dubois, E. L.:* Renal biopsy in rheumatoid arthritis with L. E. cells. Arthritis Rheum. *5:* 283–284, 1962.
3. *Brunner, C. M. & Davies, J. S.:* Characteristics of antinuclear factors in Felty's syndrome. Arthritis Rheum. *13:* 33–37, 1970.
4. *Cebecauer, L.:* Personal communication.
5. *Denko, C. W. & Zumpft, C. W.:* Chronic arthritis with splenomegaly and leucopenia. Arthritis. Rheum. *5:* 478–491, 1962.
6. *Diem, K.* (Ed.): Documenta Geigy. Scientific Tables, 6th ed. J. R. Geigy, S. A., Basle 1962, p. 36–39 and 124–127.
7. *Elling, P., Graudal, H. & Faber, V.:* Organ-specific and organ-non-specific autoantibodies in rheumatoid arthritis. Acta med. scand. *182:* 707–715, 1967.
8. *Everall, P. H. & Wright, G. H.:* Low pressure ultrafiltration of protein-containing fluids. J. Med. Lab. Technol. *15:* 209–213, 1958.
9. *Franco, A. E. & Schur, P. H.:* Hypocomplementemic rheumatoid arthritis. Arthritis Rheum. *14:* 162, 1971 (abstr.).
10. *Gell, P. G. H. & Coombs, R. R. A.:* The classification of allergic reactions responsible for clinical hypersensitivity and disease. In: *Gell, P. G. H. & Coombs, R. R. A.* (Eds.): Clinical Aspects of Immunology. 2nd ed. Blackwell Scientific Publications Ltd., Oxford 1968, p. 575–596.
11. *Gordon, D. A., Bell, D. A., Baumal, R. & Broder, I.:* Studies into the occurrence of soluble antigen-antibody complexes in disease. IV. Correlation between the rheumatoid biologically active factor and the clinical features of rheumatoid arthritis. Clin. exp. Immunol. *5:* 57–66, 1969.
12. *Hollcroft, R. M., Dubois, E. L., Lundberg, G., Chandor, S., Gilbert, S., Quismorio, F., Friou, G. & Barbour, B.:* Renal damage in systemic lupus erythematosus with normal renal function. J. Rheumatol. *1:* suppl. 1, 15, 1974 (abstr.).
13. *Hunder, G. G. & McDuffie, F. C.:* Hypocomplementemia in rheumatoid arthritis. Amer. J. Med. *54:* 461–472, 1973.
14. *Jensen, H. & Henriksen, K.:* Proteinuria in non-renal infectious diseases. Acta med. scand. *196:* 75–82, 1974.
15. *Koffler, D., Schur, P. H. & Kunkel, H. G.:* Immunological studies concerning the nephritis of systemic lupus erythematosus. J. exp. Med. *126:* 67–623, 1967.
16. *Koffler, D., Agnello, V., Thoburn, R. & Kunkel, H. H.:* Systemic lupus erythematosus: Prototype of immune complex nephritis in man. J. exp. Med. *134:* 169–179, 1971.
17. *Krishnan, C. & Kaplan, M. H.:* Immunopathologic studies of systemic lupus erythematosus. II. Antinuclear reaction of γ-globulin eluted from homogenates of isolated glomeruli of kidneys from patients with lupus nephritis. J. Clin. Invest. *46* 569–579, 1967.
18. *Larsen, S.:* Personal communication.
19. *Laurell, C.-B.:* Composition and variation of the gel electrophoretic fractions of plasma, cerebrospinal fluid and urine. Scand. J. Clin. Lab. Invest. *29:* suppl. 124, 71–82, 1972.
20. *McIntosh, R. M., Kaufman, D. B., Kulvinskas, C. & Grossman, B. J.:* Cryoglobulins. I. Studies on the nature, incidence and clinical significance of serum cryoproteins in glomerulonephritis. J. Lab. Clin. Med. *75:* 566–577, 1970.
21. *Meltzer, M., Franklin, E. C., Elias, K., McCluskey, R. T. & Cooper, N.:* Cryoglobulinemia—A clinical and laboratory study. II. Cryoglobulins with rheumatoid factor activity. Amer. J. Med. *40:* 937–956, 1966.
22. *Norberg, R.:* IgG complexes in serum of rheumatoid arthritis patients. Scand. J. Immunol. *3* 229–236, 1974.
23. *Puritz, E. M., Yount, W. J., Newell, M. & Utsinger, P. D.:* Immunoglobulin classes and IgG subclasses of human antinuclear antibodies. A correlation of complement fixation and the nephritis of systemic lupus erythematosus. Clin. Immunol. Immunopathol. *2:* 98–113, 1973.
24. *Ropes, M. V., Bennett, G. A., Cobb, S., Jacox,*

R. & Jessar, R. A.: 1958 revision of diagnostic criteria for rheumatoid arthritis. Arthritis Rheum. *2:* 16–18, 1958.

25. *Ruderman, M., Miller, L. M. & Pinals, R. S.:* Clinical and serologic observations on 27 patients with Felty's syndrome. Arthritis Rheum. *11:* 377–384, 1968.
26. *Savory, J., Pu, P. H. & Sunderman, F. W.:* A biuret method for determination of protein in normal urine. Clin. Chem. *14:* 1160–1171, 1968.
27. *Skrifvars, B., Törnroth, T., Tallqvist, G. & Ahlqvist, J.:* Goldinduced immune complex nephritis in seronegative rheumatoid arthritis. J. Rheumatol. *1:* suppl. 1, 111, 1974 (abstr.).
28. *Sørensen, A. W. S.:* The Kidney in Rheumatoid Arthritis. Thesis. University of Copenhagen. Munksgaard, Copenhagen 1966
29. *Tojo, T. & Friou, G. J.:* Lupus nephritis: Varying complement fixing properties of immunoglobulin G antibodies to antigens of cell nuclei. Science, *161:* 904–906, 1968
30. *Weisman, M. H. & Zvaifler, N. J.:* Immunologic studies of Felty's syndrome. J. Rheumatol. *1:* suppl. 1, 105, 1974 (abstr.).
31. *Wiik, A.:* Unpublished work.
32. *Wiik, A., Jensen, E & Friis, J.:* Granulocyte-specific antinuclear factors in synovial fluids and sera from patients with rheumatoid arthritis. Ann. rheum. Dis. *33:* 515–522, 1974.
33. *Wiik, A. & Munthe, E.:* Restrictions among heavy and light chain determinants of granulocyte-specific antinuclear factors. Immunology *23:* 53–60, 1972.
34. *Wiik, A. & Munthe, E.:* Complement-fixing granulocyte-specific antinuclear factors in neutropenic cases of rheumatoid arthritis. Immunology *26:* 1127–1134, 1974.
35. *Winchester, R. J., Kunkel, H. G. & Agnello, V.:* Occurrence of γ-globulin complexes in serum and joint fluids of rheumatoid arthritis patients: Use of monoclonal rheumatoid factors as reagents for their demonstration. J. exp. Med. *134:* 286–295, 1971.

Acta path. microbiol. scand. Sect. C, 83: 280-284, 1975

AGGLUTININS TO RABBIT ERYTHROCYTES IN FLUID FROM NON-KERATINIZING JAW CYSTS

NILS SKAUG

Laboratory of Oral Microbiology, Department of Microbiology, The Gade Institute, Schools of Medicine and Dentistry, University of Bergen, Bergen, Norway

Skaug, N. Agglutinins to rabbit erythrocytes in fluid from non-keratinizing jaw cysts. Acta path. microbiol. scand. Sect. C, *83: 280-284, 1975.*

Cyst fluid obtained from 21 patients agglutinated rabbit erythrocytes to titres ranging from 16 to 512 (mode titre 128). The agglutinins behaved as specific antibodies and were mainly of the IgM class though IgG agglutinins were also demonstrated. The agglutination titres of cyst fluid showed a positive correlation with the titres of autologous serum, indicating a serum origin of the cystic agglutinins. Thus agglutinins to rabbit red cells, being the first antibody demonstrated in cyst fluid, seem to be useful markers for serum derived Ig occurring in cyst fluid.

Key words: Non-keratinizing jaw cysts; agglutinins; rabbit erythrocytes.

N. Skaug, Laboratory of Oral Microbiology, Department of Microbiology, The Gade Institute, Schools of Medicine and Dentistry, University of Bergen, Bergen, Norway.

Received 21.i.75 *Accepted 7.ii.75*

Agglutinins to rabbit red cells (3, 6) *are regularly found in human serum* (11). *The agglutinating activity depends on specific* antibodies (11), mainly of the IgM class, and *the binding to the erythrocytes is a Fab reaction* (*O. Tönder,* personal communication). The origin of these natural antibodies is unknown (12). They are also present in whole human saliva (12), extracts of faeces (4) and human milk (5).

Fluid from non-keratinizing jaw cysts is rich in immunoglobulins (10, 7). The electrophoretic and immunoelectrophoretic patterns of the cystic immunoglobulins differ somewhat from those of autologous serum (8, 9) indicating local immunoglobulin production, particularly of IgG and IgA (10, 7). However, there is evidence that part of the *immunoglobulins in cyst fluid is derived from serum* (7). The evidence is strengthened by *the present study* which shows that *agglutinins to rabbit red cells* are present in *cyst fluid in titres* paralleling those of autologous *serum.*

MATERIALS AND METHODS

Cyst Fluid and Serum

Collection and handling of cyst fluid and autologous serum have been described *elsewhere* (8). Treatment with 2-mercaptoethanol (2-ME) (Fluka *AG, Buchs, Switzerland*) was performed by incubating undiluted cyst fluid and serum, or cyst fluid and serum diluted 1:2 in phosphate buffered saline (PBS, 0.15 M NaCl, 1/150 M phosphate, pH 7.2) with equal volumes of *0.2 M* 2-ME at *37° C for 30 min. In preliminary* experiments, the

agglutination titres to rabbit erythrocytes of 2-ME treated serum and of 2-ME treated serum which subsequently had been alkylated by dialysis against 0.02 M iodoacetamid in PBS for 16 h at 4° C, and dialysed overnight against PBS, were the same. Reduced specimens of cyst fluid and serum were therefore routinely titrated without previous alkylation.

Saliva

Pooled human saliva (9) was used in the experiments.

Antisera

Rabbit antisera monospecific for human IgG, IgA and IgM were purchased from Behringwerke AG (Marburg-Lahn, West Germany). When used in inhibition experiments or in antiglobulin tests, the antisera were absorbed before use with packed rabbit erythrocytes (antiserum:erythrocytes = 2:1) to remove possible iso-agglutinins (4).

Rabbit Erythrocytes

Erythrocytes were obtained from blood collected in Alsever's solution. Before use, the erythrocytes were washed 3 times in 10 volumes of PBS and finally packed at 1000 × *g* for 10 min.

Agglutination of Rabbit Erythrocytes

The agglutination tests were performed essentially as described by *Tönder et al.* (1967). To 0.1 ml twofold dilutions in tubes of inactivated (56° C, 30 min) cyst fluid, serum or unconcentrated saliva was added 0.1 ml of a 1 per cent suspension of washed rabbit erythrocytes. The tubes were left at room temperature for 1 h and the agglutinations recorded following centrifugation for 45 sec at 1000 × *g*. Pairs of cyst fluid and autologous serum were titrated simultaneously using the same batch of rabbit erythrocytes. From replicate determinations of a series of sera, the inaccuracy in determination of the highest sample dilution giving definite agglutination was found to be ± 1 dilution

Inhibition of Agglutination

For inhibition of agglutination, 2-ME treated cyst fluid and serum, and untreated cyst fluid and serum diluted similarly (*i.e.* 1:2) in PBS were mixed with equal volumes of inhibitor (antiserum to IgM, IgA and IgG) or absorbed normal rabbit serum (control). The tubes were incubated at room temperature for 20 min, thereafter overnight at 4° C. Titration of the mixtures, addition of rabbit erythrocytes, incubation and recording of the agglutination were performed the following day as described for the agglutination test. 2-ME treated cyst fluid and serum were also diluted twofold and 4 inhibiting units of anti-IgA serum were added to each dilution and examined for inhibition of agglutination of rabbit red cells. One inhibiting unit of anti-IgA serum was defined as the highest dilution of the antiserum inhibiting the agglutination of rabbit red cells by 4 agglutinating units of saliva. One agglutinating unit of saliva was the highest saliva dilution showing definite agglutination of the rabbit erythrocytes in the test system used. As shown earlier (12), whole human saliva contains only IgA agglutinins.

Antiglobulin Test

Specimens of cyst fluid and human serum were titrated as described against rabbit red cells and left at room temperature for 1 h. After 3 washings in PBS, 0.1 ml of anti-IgG serum, pretested for antiglobulin activity against 1 per cent commercial human IgG (AB Kabi, Stockholm, Sweden) and diluted 1:80 in PBS, was added to each tube. The racks were shaken and left at room temperature for 1 h before recording the agglutinin titres. In preliminary tests against human serum, a dilution of 1:80 of the anti-IgG serum gave maximum rise in titre. In all experiments, a similar dilution of normal rabbit serum served as a control.

Absorption of Cyst Fluid and Serum

Cyst fluid or serum diluted 1:4 in PBS were mixed with an equal volume of packed rabbit erythrocytes, incubated for 30 min at room temperature and made cell-free by centrifugation at 1000 × *g* for 5 min.

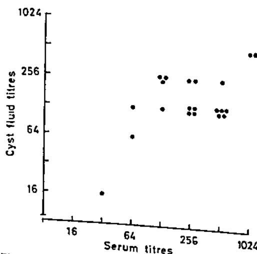


Fig. 1. Distribution of titres of agglutinins to rabbit erythrocytes in cyst fluid and autologous serum.

TABLE 1. *Titres of Agglutinins to Rabbit Erythrocytes in 12 Pairs of Cyst Fluid and Autologous Serum before and after Reduction with 0.2 M 2-mercaptoethanol (2-ME)*

Types of cysts	Untreated		2-ME treated	
	Cyst fluid	Serum	Cyst fluid	Serum
Apical periodontal	256	128	16	16
Apical periodontal	256	256	16	32
Apical periodontal	64	128	8	16
Apical periodontal	128	256	8	16
Apical periodontal	256	256	128	64
Apical periodontal	64	256	<8	32
Apical periodontal	128	256	64	32
Follicular	16	256	8	16
Follicular	256	512	8	32
Globulomaxillary	256	512	64	32
Residual	512	1024	16	8
Residual	512	1024	16	64

Single Radial Immunodiffusion

Immuno-Plates® (Hyland Div. Travenol Lab., Costa Mesa, Calif., USA) were used for quantitation of IgG, IgA and IgM in cyst fluid and serum.

Statistics

The significance of the differences between corresponding agglutination titres of cyst fluid and serum was determined by the Wilcoxon test for pair differences (1). For evaluation of the relationship between the titres of cyst fluid and autologous serum, the Spearman's rank correlation test was used (1).

RESULTS

Occurrence of Agglutinins to Rabbit Erythrocytes

Serum and cyst fluid from 21 patients were tested. In cyst fluid, the titres ranged from 16 to 512 (mode titre 128) and in serum from 32 to 1024 (mode titre 256) (Fig. 1). The differences between corresponding titres of cyst fluid and serum were statistically significant ($0.01 < P < 0.02$). Also, a statistically significant positive correlation between the titres of cyst fluid and autologous serum could be established ($R = +0.5820$, $P < 0.01$). A significant and similar drop in titre was obtained after treatment of 12 pairs of cyst fluid and autologous serum with 2-ME (Table 1). In 20 pairs of cyst fluid and autologous serum examined, the cyst fluid to autologous serum ratios for agglutinins to rabbit erythrocytes differed from the ratios for concentrations of Ig (Table 2).

Antiglobulin test with anti-IgG serum diluted 1:80 in PBS augmented the agglutination titre of 2 cyst fluids examined by 3 and 4 titre steps, respectively. In 5 human sera, the agglutinating activity of the IgG agglutinins was increased by 3 to 5 steps using the same dilution of the anti-IgG serum.

Incubation of untreated cyst fluid and serum from 5 patients with anti-IgM serum inhibited the agglutination of rabbit red cells by 3 or 4 steps whereas no inhibition was recorded after incubation with anti-IgA or anti-IgG serum. Antiserum to IgM had no inhibitory effect on 2-ME treated cyst fluid or serum. Nor was the agglutination of rabbit red cells by the 2-ME treated cyst fluids or sera, undiluted or diluted twofold, inhibited by anti-IgA serum.

Supernatants collected after absorption of cyst fluid and serum with packed rabbit erythrocytes contained no detectable agglutinins to rabbit erythrocytes

DISCUSSION

Agglutinins to rabbit erythrocytes were found in all pairs of cyst fluid and serum examined.

TABLE 2. *Ratios of Titres of Agglutinins to Rabbit Red Cells and of Immunoglobulin Levels in 20 Pairs of Cyst Fluid and Autologous Serum*

Types of cysts		Cyst fluid/serum ratios		
	Agglutinins*	IgG	IgA	IgM
Apical periodontal	0.50	2.50	8.8	1.50
Apical periodontal	0.50	1.08	1.19	0.62
Apical periodontal	2.00	1.08	1.47	0.83
Apical periodontal	0.50	1.51	2.71	0.78
Apical periodontal	1.00	1.41	1.36	1.23
Apical periodontal	1.00	1.26	2.14	1.00
Apical periodontal	0.25	3.44	1.47	0.95
Apical periodontal	1.00	1.07	0.90	0.90
Apical periodontal	0.50	0.79	1.55	1.51
Apical periodontal	0.25	1.31	2.98	1.37
Apical periodontal	0.25	1.37	1.07	0.64
Apical periodontal	0.25	1.44	1.79	1.00
Follicular	2.00	0.68	0.45	0.80
Follicular	0.50	2.44	1.31	0.57
Follicular	0.50	1.40	1.71	0.93
Globulomaxillary	0.50	1.61	1.50	1.17
Median palatine	0.50	1.25	3.17	0.36
Residual	2.00	1.05	1.46	1.75
Residual	0.33	1.67	3.20	1.28
Residual	2.00	1.67	3.20	1.28

* Ratios of the reciprocal of titres.

The sensitivity of the agglutinins to treatment with 2-ME and the results of the inhibition tests showed that the agglutinins in cyst fluid mainly belonged to the IgM class. Nevertheless, the results of the antiglobulin test showed that, as in serum (11), IgG agglutinins are responsible for some of the agglutinating activity of cyst fluid. Although anti-IgA serum did not inhibit the agglutination of rabbit red cells, small amounts of IgA agglutinins may still be present in cyst fluid.

The statistically significant correlation between the agglutinin titres of cyst fluid and serum together with the similar effect of 2-ME on the agglutinating activity of cyst fluid and serum and the results of the inhibition studies strongly indicate that cystic agglutinins to rabbit red cells originate from serum. A corresponding parallelism in agglutinin titres of normal as well as of pathological spinal fluid and autologous serum (2), and of human milk and autologous serum (5) has been observed.

The finding that the cyst fluid to autologous serum ratios for agglutinins mostly were <1 and the corresponding Ig ratios mostly were >1 indicates some restriction of the passage of immunoglobulins from the blood to the cyst cavity. It also suggests that the high concentrations of Ig in cyst fluid are not solely due to a selective transport of serum derived Ig across the cyst capsule.

Although the present investigation does not indicate a local production of cystic agglutinins to rabbit red cells this cannot be completely disregarded. Thus secretory IgA of saliva (12), of faeces (4) and of human colostrum (5) agglutinate erythrocytes from rabbits. However, the oral cavity and the large intestine both harbour a complex microflora which may well contain antigens stimulating the production of agglutinins cross-reacting with rabbit red cells. In contrast, fluid from jaw cysts is most often sterile (8).

In conclusion, antibodies against rabbit erythrocytes appear to be useful markers for serum Ig leaking into and accumulating in

jaw cysts. They are also the first specific antibodies to be demonstrated in fluid from such cysts. Further investigations concerning antibody activity in cyst fluid are in progress.

REFERENCES

1. *Documenta Geigy Scientific Tables*, Diem, K. (ed.), 6th ed. J. R. Geigy, S. A., Basel (Switzerland) 1962, p. 180, 191–192.
2. *Fossan, G. & Tönder, O.:* The blood-cerebrospinal fluid barrier function studied by "natural" marker antibodies. Scand. J. Immunol. *3:* 876, 1974 (Abstract).
3. *Friedberger, E., Bock, G. & Fürstenheim, A.:* Zur Normalantikörperkurve des Menschen durch die verschiedenen Lebensalter und ihre Bedeutung für die Erklärung der Hautteste (Schick, Dick)[1]. Z. Immun-Forsch. *64:* 294–319, 1929.
4. *Haneberg, B.:* Human fecal agglutinins to rabbit erythrocytes. Scand. J. Immunol. *3:* 71–76, 1974.
5. *Haneberg, B.:* Human milk immunoglobulins and agglutinins to rabbit erythrocytes. Int. Arch. Allergy *47:* 716–729, 1974.
6. *Schiff, F.:* Heterogenetic hemagglutinins in man following therapeutic injections of immune sera produced in rabbits. J. Immunol *33:* 305–313, 1937.
7. *Skaug, N.:* Proteins in fluid from non-keratinizing jaw cysts. 4. Concentrations of immunoglobulins (IgG, IgA and IgM) and some non-immunoglobulin proteins: Relevance to concepts of cyst wall permeability and clearance of cystic proteins. J. oral. Path. *3:* 47–61, 1974.
8. *Skaug, N. & Hofstad, T.:* Proteins in fluid from non-keratinizing jaw cysts. 1. Separation patterns on cellulose acetate membranes and percentage distribution of the electrophoretic fractions. J. oral. Path. *2:* 112–125, 1973.
9. *Skaug, N. & Hofstad, T.: Immunoglobulins in* fluid from non-keratinizing jaw cysts. Scand. J. Immunol. 1975, in press.
10. *Toller, P. A. & Holborow, E. J.:* Immunoglobulins and immunoglobulin-containing cells in cysts of the jaws Lancet *ii:* 178–181, 1969.
11. *Tönder, O., Natvig, J. B. & Matre, R.:* Antibodies in human sera to rabbit erythrocytes. *Immunology 12* 629–637, 1967.
12. *Tönder, O. & Larsen, B* Agglutinins in human saliva to rabbit erythrocytes. Vox Sang. *24:* 515–523, 1973

Acta path. microbiol. scand. Sect. C, *83:* 285–288, 1975

COMPLEMENT ACTIVATION IN MENINGOCOCCAL SEPTICEMIA

ULF JOHNSON and ANNA-BRITA LAURELL

Institute of Medical Microbiology, University of Lund, Lund, Sweden

Johnson, U. & Laurell, A.-B. Complement activation in meningococcal septicemia. Acta path. microbiol. scand. Sect. C, *83:* 285–288, 1975.

Analyses of complement components in plasma from a patient with meningococcal septicemia showed abnormally low levels of C3 and properdin. C4 remained within the normal level. Activation of C3 proactivator and conversion of C3 was shown. Except these findings, which are in agreement with activation of complement by endotoxin by the alternate pathway, a marked decrease in C1q was observed.

Key words: Complement activation; meningococcal septicemia.

Ulf Johnson, Institute of Medical Microbiology, Sölvegatan 23, S-223 62 Lund, Sweden.

Received 31.i.75 Accepted 31.i.75

Endotoxin is known to activate complement (13). The lipopolysaccharide interacts with properdin (P), a basic euglobulin occurring in normal human serum in a concentration of about 25 µg/ml (7). Properdin is a component of the alternate pathway of complement. Activation leads to consumption of the complement components C3 to C9 bypassing the components C1 to C4 of the classical activation pathway (6). Interaction with lipopolysaccharide converts P to its active form, P. This conversion is thought to be mediated by properdin convertase, an α-globulin present in normal human serum (16).

The activated properdin enzymatically cleaves C3 to C3a and C3b (8). Together with C3-proactivator convertase (C3PAse) C3b cleaves C3-proactivator (C3PA) into C3-activator (C3A), an enzyme capable of splitting native C3 into C3a and C3b. Thus, C3b initiates a positive feedback leading to further cleavage of C3 into C3a and C3b (7). C3b participates also in the activation of the terminal complement components C5 to C9. In normal serum C3b is inactivated by the C3b-inactivator (C3b-INA), an enzyme splitting C3b into C3c (β_{1A}) and C3d (α_{2D}) (14). C3a is an anaphylatoxin, which is a potent liberator of histamine, and it also has a chemotactic effect on neutrophile granulocytes (4). In normal serum C3a is inactivated by a carboxypeptidase B like enzyme, the anaphylatoxin inactivator (AI) (2).

In vitro incubation of fresh human serum with endotoxin leads to activation of the alternate pathway of the complement system (6). In an endeavour to elucidate the mechanism of complement activation observed in endotoxemia (12) we analysed the complement components C1q, C4, C3, C3PA, C5 and P in a case with severe meningococcal septicemia.

CASE REPORT

An 11 year girl was admitted in pre-shock to hospital because of assumed bacterial meningitis

C.S.F. obtained on admission contained intracellular gram-negative diplococci. Culture gave growth of *meningococci. A few hours after admission* skin lesions typical of meningococcemia developed and culture of the blood gave growth of meningococci. Treatment with sulfonamide, penicillin and chloramphenicol was started immediately. The patient *improved until the third day*, when signs of pericarditis and pleurisy with effusion appeared. Aspiration of the pericardial and pleural effusions and treatment with antibiotics was followed by further improvement of the patient who left hospital without sequely.

MATERIALS AND METHODS

Blood samples for complement analyses were obtained *immediately after admission to hospital.* Two additional blood samples were collected three and five days later. The samples were collected both as serum and EDTA-plasma. The samples were immediately centrifuged and frozen in aliquots *at −80° C until analysed.*

Electrophoresis. The complement components C1q, C4, C3, C5 and P were quantitated by the *electroimmuno assay* (9) and the results were noted as percentages of a normal standard. *Conversion of C3 was measured by crossed immunoelectrophoresis* (10, 5). Conversion of C3PA to C3A was assessed with *immunoelectrophoresis.* All the analyses were made on EDTA-plasma.

Buffers. All the quantitative determinations, *except of C1q and C3PA, were made* with 0.075 M barbital buffer, pH 8.6, containing no Ca^{++}. Immunoelectrophoresis on C3PA was done with 0.075 M barbital buffer, pH 8.3, containing Na_2EDTA 2×10^{-3} M. C1q was quantitated by electroimmuno assay using a phosphate buffer, pH 6.0, ionic strength 0.1 with a voltage of 5 V/cm applied over the plate for 20 hours.

Error of the method. The calculated error of the electroimmuno assay (2SD) was about ± 10 per cent (15)

Standard pools of plasma and serum. Aliquots *of plasma and serum from 100 apparently healthy* blood donors were pooled and used as standard plasma and serum pools, respectively. The standards were stored in small volumes at −80° C. The pools were defined as containing 100 per cent of the complement component determined. The normal ranges used for C1q, C4, C3 and C5 are those given by *Sjöholm* (15). *Plasma from 100 apparently* healthy blood donors were analysed for properdin. The normal range for properdin was found to be 50 to 150 per cent of the normal standard pool.

Antisera. Specific antisera against *C1q, C4, C3, C3PA* and *C5* were prepared as described by *Sjöholm* (15). Antiserum specific to *P was raised as* described previously (8).

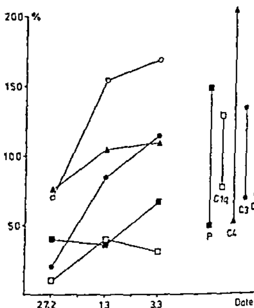


Fig. 1. Levels of the complement components C1q, C4, C3, C5 and P in a case with meningococcal septicemia. The normal range of each component is given to the right.

RESULTS

During the acute phase of illness C1q, C3, C5 and P were abnormally low (Fig. 1). Fragmentation products (C3c) from C3 were observed (Fig. 2) and C3PA was converted *into* C3A. The level of C4 remained within the normal range.

By the third day of illness all components determined, *with the exception* of C1q, had returned to normal levels. Cleavage products of C3 were still present and conversion of C3PA was demonstrated.

Five days after the acute phase C1q and P were still subnormal. However, on crossed immunoelectrophoresis C3 appeared *in the unconverted form* and conversion of C3PA into C3A was no longer demonstrable.

DISCUSSION

Recently *McCabe* reported abnormally low C3 levels in gram-negative bacteremia complicated by shock (12). Shock or a fatal outcome tended to be more frequent in cases where C3 was low in samples collected short-

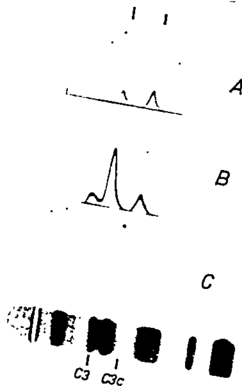


Fig. 2 Crossed immunoelectrophoresis on plasma from a case with meningococcal septicemia showing conversion of C3. The gel contains monospecific antibodies to C3. Anode is to the right.
A Acute sample.
B Sample obtained on the third day of illness.
C. Sample obtained on the fifth day of illness. The electrophoretic pattern of normal human plasma in agarose gel is given as reference.

ly after the onset of bacteremia. In our case of meningococcal septicemia C1q, C3, C5 and P, but not C4, were depressed below the lower limit of the normal range. In addition, conversion of C3 and formation of C3A from C3PA were detected in the acute phase samples. The analyses were made on EDTA-plasma, which was properly handled (see Methods), and thus indicated *in vivo* changes of the complement.

Low C3 levels in connection with the formation of C3 fragments (C3c), conversion of C3PA into C3A and low levels of properdin are findings suggesting activation of the alternate pathway of the complement system. The markedly reduced C1q value together with a C4 level within the normal range is incompatible with concomitant complement activation by the classical pathway. The pronounced deviation of the values for these two complement factors in the course of the illness also suggests involvement of hithero unknown mechanisms. However, *Loos et al.* recently reported an interaction between C1q and lipopolysaccharides (11), a finding which might help to explain the low C1q levels. On treatment C3 and C5 rapidly normalized and on the fifth day the C5 value bordered the upper limit of the normal range. This was accompanied by the appearance of a moderate increase in C4. Normalization of properdin, however, was much slower.

The rapidly rising levels of C3 and C5 on cessation of activation and consumption might be explained by an excessive *de novo* synthesis or by their behaviour as acute phase reactants. C3 belongs to the acute phase reactants and probably also C4 (1).

In a study of the Dengue hemorrhagic shock syndrome *Bokisch et al.* (3) produced evidence that the massive complement activation caused the shock by generation of the anaphylatoxins and by initiation of intravascular coagulation. Complement activation with liberation of the anaphylatoxins might be responsible for the shock symptoms in severe infections with gram-negative bacteria, but substantiating evidence is still lacking. Dysfunction of the anaphylatoxin inactivator or of C3bINA might be one explanation for a defective inactivation of C3a, C5a or C3b formed during activation of the complement by endotoxin. However, the generation of anaphylatoxins on complement activation in gram-negative septicemia might be too rapid and excessive for the normal capacity of the anaphylatoxin inactivator and thereby lead to shock.

Determination of C3, C4 and C3PA may be a ready aid in the clinical management of suspect severe gram-negative infections. The finding of a low C3 value in association with conversion of C3, conversion of C3PA into

C3A and a normal C4 value lend support to a diagnosis of a severe endotoxemia. With the aid of electroimmuno assay and the crossed immunoelectrophoresis these determinations can be made within 4 to 6 hours.

This work was supported by grants from the *Swedish Medical Research Council* (Grant B75-16X-68-11A), from *Alfred Österlunds Stiftelse* and from the *Medical Faculty, University of Lund.*

REFERENCES

1. *Aronsen, K.-F., Ekelund, G., Kindmark, C.-O. & Laurell, C.-B.:* Sequential changes of plasma proteins after surgical trauma. Scand. J. Clin. Lab. Invest. *29:* suppl. 124: 127–136, 1972.
2. *Bokisch, V. A. & Müller-Eberhard, H. J.:* Anaphylatoxin inactivator of human plasma: Its characterization as a carboxypeptidase. J. Clin. Invest. *49:* 2427–2436, 1970.
3. *Bokisch, V. A., Top, F. H., Russel, P. K., Dixon, F. J. & Müller-Eberhard, H. J.:* The potential pathogenic role of complement in Dengue hemorrhagic shock syndrom. N. Engl. J. Med. *289:* 996–1000, 1973.
4. *Bokisch, V. A., Müller-Eberhard, H. J. & Cochrane, C. G.:* Isolation of a fragment (C3a) of the third component of human complement containing anaphylatoxin and chemotactic activity and description of an anaphylatoxin inactivator of human serum. J. Exp. Med. *129:* 1109–1130, 1969.
5. *Ganrot, P. O.:* Crossed immunoelectrophoresis. *Scand. J. Clin. Lab. Invest. 29: suppl. 124:* 39–47, 1972.
6. *Gewurz, H., Shin, H. S. & Mergenhagen, S. E.:* Interactions of the complement system with endotoxic lipopolysaccharide: Consumption of each of the six terminal complement components. J. Exp. Med. *128:* 1049–1057, 1968.
7. *Götze, O. & Müller-Eberhard, H. J.:* The role of properdin in the alternate pathway of complement activation. J. Exp. Med. *139:* 44–57, 1974.
8. *Johnson, U.:* Properdin acting as a C3-convertase. Acta path. microbiol. scand. Sect. B, *82.* 914–916, 1974.
9. *Laurell, C.-B :* Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Anal. Biochem. *15:* 45–52, 1966.
10. *Laurell, C.-B.:* Antigen-antibody crossed electrophoresis. Anal. Biochem. *10:* 358–361, 1965.
11. *Loos, M., Bitter-Suerman, D. & Dierich, M.:* Interaction of the first (CI), the second (C2) and fourth *(C4) component of complement* with different preparations of bacterial lipopolysaccharides and with lipid A. J. Immunol. *112:* 935–940, 1974.
12. *McCabe, W.:* Serum complement levels in bacteremia due to gram-negative organisms. N. Engl. J. Med. *288:* 21–23, 1973.
13. *Pillemer, L., Schoenberg, M., Blum, L. & Wurz, L.:* Properdin system and immunity. II. Interaction of the properdin system with polysaccharides. Science *122:* 545–549, 1955.
14. *Ruddy, S. & Austen, K. F.:* C3b inactivator of man. II. Fragments produced by C3b-inactivator cleavage of cell-bound or fluid phase C3b. J. Immunol. *107:* 742–750, 1971.
15. *Sjöholm, A.:* Complement components in *normal serum* and plasma quantitated by electroimmuno assay. Scand J. Immunol. In press
16. *Stitzel, A & Spitzer, R.:* The utilization of properdin in the alternate pathway of complement activation· Isolation of properdin convertase J. Immunol. *112* · 56–62, 1974.

Acta path. microbiol. scand. Sect. C, *83:* 289–297, 1975

THE IMMUNOLOGICAL POTENTIAL OF HUMAN LYMPH NODES IN UREMIA AND DURING EXTRACORPOREAL IRRADIATION OF THE BLOOD

S. A. Birkeland, J. Moesner, F. Amtrup and E. V. Svendsen

Tissue Culture Laboratory, Institute of Pathology, Dept. of Nephrology and Dept. of Thoracic Surgery, Odense University Hospital, DK-5000 Odense, Denmark

Birkeland, S. A., Moesner, J., Amtrup, F. & Svendsen, E. V. The immunological potential of human lymph nodes in uremia and during extracorporeal irradiation of the blood. Acta path. microbiol. scand. Sect. C, *83:* 289–297, 1975.

15 inguinal lymph nodes from uremic patients were compared with 15 lymph nodes from normal patients and with 15 lymph nodes from the same uremic patients after extracorporeal irradiation of the blood. The parameters recommended by WHO for reporting lymph node morphology in relation to immunological function were used to evaluate the lymph nodes. Pairs of lymph nodes were compared in a random series. Preparations for light microscopy were evaluated objectively and independently by two observers in two separate examinations, so that each lymph node pair was evaluated four times. The observations made by the same person were found to be consistent and the two observers' evaluations to be in good agreement. The results of the comparisons were given a rank value and the average rank value for each parameter calculated from the values for each pair. The results for each parameter were evaluated using Wilcoxon's rank-sum test of pair differences. In uremic lymph nodes a reduction was found in the number of germinal centres and the density of lymphocytes in the paracortex, and after ECIB the lymph nodes showed a tendency to an increase in all the examined parameters.

Key words: Human lymph nodes; immunological potential; uremia; extracorporeal irradiation.

S. A. Birkeland, Kløverværnget 26 C, DK-5000 Odense, Denmark.

Received 16.ix.74 Accepted 13.ii.75

"Observing the hard fact of unreliability of repeated clinical measurements is a humbling experience, and one highly recommended" (*Blackburn et al.* 1960).

Lymph nodes play a prominent part in the immune system of the organism and are important for both the cellular and the humoral immune response.

The purpose of the present study is to make a controlled evaluation of the immunological status of lymph nodes during uremia, when immunocapacity is depressed generally, and after extracorporeal irradiation of the blood (ECIB), when in particular the number of recirculating lymphocytes is selectively reduced, without any effect on the remainder of the immune system.

The structure of lymph nodes has been the subject of many studies (20, 22, 23). The

TABLE 1. *Patient Material*

No.	Sex	Age (years)	Diagnosis	Type of ECIB*	Accumulated dose (rad)	No. of blood volumes irradiated
1	M	23	chr. glom. nephr.	137-Cs	49,999	126
2	M	31	heridit. nephr.	137-Cs	50,050	133
3	F	38	chr. glom. nephr.	137-Cs	50,063	134
4§	M	40	chr. glom. nephr.	137-Cs	(34,898)	(84)
5	M	47	nephrosclerosis	137-Cs	50,077	133
6	F	43	chr. glom. nephr.	137-Cs	50,005	132
7	M	46	chr. pyelonephr.	137-Cs	50,025	135
8	F	38	chr. glom. nephr.	250-kV	38,704	154
9	M	54	chr. glom. nephr.	250-kV	36,951	176
10	M	61	chr. glom. nephr.	250-kV	38,220	210
11	M	35	oxalosis	250-kV	37,325	107
12	M	36	chr. glom. nephr.	250-kV	36,252	104
13	M	40	chr. glom. nephr.	90-Sr	5,114	181
14	F	52	chr. glom. nephr.	90-Sr	5,357	234
15	F	27	chr. glom. nephr.	90-Sr	5,487	190

§ In a patient No. 4 both lymph nodes were removed before extrocorporeal irradiation (ECIB) and this patient is therefore only included in consistency evaluations.

* A more detailed account of the ECIB treatment used is given elsewhere (1). The 3 patients treated with the beta-emitter were included in the study, because both the depletion of the recirculating lymphocytes and the no. of irradiated blood volumes were of the same order of magnitude as in the 12 patients treated with the other methods. If they are excluded the conclusions are the same.

terminology recommended by the World Health Organisation (WHO) (5) is used in this study.

MATERIAL AND METHODS

The material consisted of 45 lymph nodes: 15 from normal patients, 15 from uremic patients before ECIB, and 15 from the same patients after this treatment.

Superficial inguinal lymph nodes were used, as these are the only practical possibility in a systematic investigation in which lymph nodes are to be removed from the same patient on two separate occasions. The disadvantage with these lymph nodes is that they drain peripheral areas in which infections are often localised. During excision the tissue was manipulated as little as possible in order to avoid the production of spurious inflammatory changes.

The normal material was removed from patients undergoing operation for inguinal hernia. The use of lymph nodes from donors after unexpected and sudden death had to be abandoned because post-mortal autolytic changes allowed these lymph nodes to be clearly distinguished from others, so that objective comparisons were impossible. The normal patients comprised 12 men and 3 women with an average age of 51 years.

The uremic patients were 15 persons treated with ECIB in the course of hemodialysis, in preparation for transplantation at a later date.* The sex, age, diagnosis and type of ECIB treatment given are shown in Table 1. Lymph nodes were removed as near to the start and finish of ECIB treatment as possible, where practicable in conjunction with other necessary operations, such as the creation of an arterio-venous fistula. In one case the second lymph node was excised during kidney transplantation.

A more detailed account of the ECIB treatment used has been given earlier (1). In the 15 patients included in this study a marked depletion of the lymphocytes was always seen after ECIB.

Evaluation of Lymph Nodes

All the lymph nodes removed were sent to the laboratory for routine examination. In the routine procedure, a varying number of sections were taken and all of these sections were subsequently used in this study. Thus the material compared was not specially selected and all the available material was included in the evaluations described below.

* The patients had been in hemodialysis for a long period before entering this trial, and there were no alterations in this treatment during the trial. Nobody received immunosuppressive treatment besides the ECIB.

TABLE 2. *Parameters Used in the Evaluation of Lymph Nodes*

1) Total amount of lymphoid tissue.
2) Number of germinal centres.
2a) Density of lymphocytes in mantle zone around germinal centres.
2b) Size of mantle zone in relation to germinal zone.
3) Density of lymphocytes in cortex.
4) Density of lymphocytes in paracortex.
5) Density of lymphocytes in medullary cords.
6) Amount of phagocyted material.
7) Amount of plasma cells.

The term "total amount of lymphoid tissue" means a subjective evaluation of the relative amount of lymphoid tissue in the lymph node.

Lymph nodes were examined using conventional light microscopy and evaluated using the parameters listed in Table 2. Normal lymph nodes were compared with lymph nodes taken from uremic patients before ECIB treatment. The latter were also compared with lymph nodes removed from the same patients after ECIB treatment. These two series of comparisons were made independently. Thus lymph nodes were compared in pairs, each pair consisting either of normal and uremic lymph nodes, or of lymph nodes before and after ECIB. The pairs were ordered randomly so that in some cases the normal lymph node (or lymph node before ECIB) was the first of a pair, while in others it was second. The pairs were evaluated by two observers (A and B). After the first evaluation (suffix 1) the random arrangement of pairs was repeated and a second evaluation (suffix 2) made. The preparations were coded so that both evaluations were made blind. In this

TABLE 3. *Results of Evaluations of Lymph Node Pairs Normal/Uremic*

Parameter	1				2				2a				2b				3				4				5				6				7			
lymph node pairs*	A1	A2	B1	B2	A1	A2	B1	B2	A1	A2	B1	B2	A1	A2	B1	B2	A1	A2	B1	B2	A1	A2	B1	B2	A1	A2	B1	B2	A1	A2	B1	B2	A1	A2	B1	B2
1-2	=	<	<	<	>	>	>	>	=	=							<	<	=	=	>	>	=	>	>	>	>	>	>	>	>	>	=	=	=	=
3-4	<	<	<	<	<	<	=	=									<	<	<	<	<	<	<	<	=	=	>	=	=	=	=	=	=	>	=	=
5-6	>	>	>	>	=	=	>	=									>	>	>	<	>	=	>	=	>	>	>	<	=	=	=	=	<	=	=	=
7-8	=	<	<	<	<	<	=	<	>	>	>	>				>	>	>	>	>	>	>	>	>	>	>	=	<	<	=	=	=	=	=	<	<
9-10	>	>	>	>	>	>	>	>									>	>	>	>	>	>	>	>	>	>	>	>	=	=	=	=	<	=	<	>
11-12	<	<	<	<	>	>	>	>	>	>	>	>					=	>	>	>	>	>	>	>	<	>	<	<	=	<	=	=	=	=	=	=
13-14	>	>	>	>	<	<	<	<		<							>	<	>	<	=	<	=	<	<	<	<	<	<	=	=	=	>	<	>	=
15-16	>	>	>	>	>	>	>	>									<	<	<	=	>	>	>	>	<	>	=	<	<	<	>	>	<	>	=	=
17-18	>	=	<	<	>	>	>	>									>	=	=	=	>	>	=	>	>	=	>	>	>	<	=	=	=	=	=	<
19-20	<	<	<	<	<	<	=	<	>	>	>	>			>	>	>	>	>	>	>	>	=	<	>	>	<	<	<	>	=	=	>	<	>	=
21-22	<	<	<	<	>	>	=	=	>	<	<	<			<	<	=	<	=	<	=	=	=	<	<	<	<	<	<	>	=	=	>	<	=	=
23-24	>	>	>	>	>	>	=	=									<	=	=	<	>	>	>	=	=	>	<	>	>	>	>	=	>	=	=	=
25-26	>	>	>	>	>	>	>	>									>	>	>	>	>	>	>	>	>	>	>	>	>	>	<	=	<	<	>	=
27-28	>	>	>	>	>	>	>	>									<	>	>	>	=	=	=	<	<	>	>	>	<	<	>	>	>	<	=	>
29-30	>	>	>	>	>	>	>	>									>	>	>	>	>	>	>	>	>	>	>	>	=	>	>	>	=	=	>	>

< parameter greater in uremic than in normal lymph nodes.
= parameter of equal size in both uremic and normal lymph nodes.
> parameter smaller in uremic than in normal lymph nodes.
☐ no comparison made.

A, B two observers
1, 2 two separate evaluations.
* The pairs were evaluated blind and in a random series.

TABLE 4. *Evaluation of c*

Parameter		1		2		2a	
Results compared		I	II	I	II	I	II
A1 and A2	intraobserver	12	15	15	15	4/5	4/5
B1 and B2	consistency	15	15	12	15	4/4	4/4
A1 and B1	interobserver	12	14	9	15	3/4	3/4
A2 and B2	consistency	14	15	12	15	4/4	4/4
A1, A2, B1 and B2		12	14	9	15	3/4	3/4

The total number of pairs compared was 15, except for parameters 2a and 2b where the total number compared is given as the denominator of a fraction.
Column I. Number of pairs in which evaluations are in full agreement.

way 4 separate evaluations were made of each pair.

For each pair, each parameter of the first lymph node was compared with that of the second and the evaluation classified into three types: change from smaller to larger (<), no change (=), or change from larger to smaller (>). These results are presented in Tables 3 and 4.

The degree of agreement (consistency) was assessed both for the individual observer in the two evaluations (intra-observer consistency) and between the two observers (inter-observer consistency). For the latter the assessment was made on the basis of the first evaluation alone, the second evaluation *alone and on both evaluations together*. The criterion for consistency was either full agreement with all signs the same (columns I, Tables 4 and 7) or partial agreement with all signs either > and =, or < and = (columns II, Tables 4 and 7—number of pairs with either full or partial agreement).

So that the comparisons could be subjected to a statistical treatment, each comparison was given a rank value; < was given rank value +1, = was given 0 and > given −1. The size of the rank values is statistically irrelevant, since the object is to obtain average values which can be compared. The average rank values for each parameter (R) and the level of significance (p) calculated using Wilcoxon's rank-sum test of pair differences are given in Tables 5 and 8.

RESULTS

1) *Normal – Uremic Patients*

The results of the comparisons and their consistency are shown in Tables 3 and 4 respectively. The statistical evaluation of the results is given in Table 5. It can be seen that the results are highly consistent. $\overline{R}$, the average rank value is negative for all parameters, compared with normals all the parameters of uremic lymph nodes show a tendency towards a reduction. This tendency might of course be fortuitous, but is statistically significant for number of germinal centres and for lymphocyte density in the paracortex ($p = 0.05–0.02$). Within the limitations of the technique used, in can be concluded that both the cell-mediated and the humoral capacity are reduced in uremic lymphnodes.

2) *Before – After ECIB*

The results of the comparisons and their consistency are shown in Tables 6 and 7 respectively. The statistical evaluation of the

TABLE 5 *Statistical Evaluation of Comparison Normal/Uremic Lymph Nodes*

ameter		1	2	2a	2b	3	4	5	6	7
coxon test of	R	−0.6	−1.66	(−1.80)	(−0.33)	−1.07	−1.86	−1.07	−1.14	−0.-
: differences	p	» 0.1	0.05–0.02	–	–	» 0.1	0.05–0.02	» 0.1	» 0.1	» 0.

$\overline{R}$ average rank value (see text).
p probability, Wilcoxon test of pair differences.

mal and Uremic Lymph Nodes)

2b		3		4		5		6		7	
I	II	I	II	I	II	I	II	I	II	I	II
/2	2/2	9	13	13	15	9	12	8	12	6	9
/2	2/2	10	13	7	15	10	13	13	15	9	14
–	–	10	14	11	15	9	13	6	12	7	14
–	–	10	14	11	14	8	10	7	13	4	14
–	–	6	11	7	14	6	8	4	9	2	8

Column II. Number of pairs in which evaluations are either in full agreement or in partial agreement (see text).

TABLE 6. *Results of Evaluations of Lymph Node Pairs Before/After ECIB*

Parameter	1				2				2a				2b				3				4				5				6				7			
lymph* node pairs	A1	A2	B1	B2	A1	A2	B1	B2	A1	A2	B1	B2	A1	A2	B1	B2	A1	A2	B1	B2	A1	A2	B1	B2	A1	A2	B1	B2	A1	A2	B1	B2	A1	A2	B1	B2
1–2	<	<	<	<	<	<	<	<	>	>	-	<					-	>	-	<	>	>	>	<	>	>	<	-	<	<	<	<	<	>	<	<
3–4	<	<	<	<	-	-	-	<									<	<	<	<	<	<	<	<	<	<	<	<	-	<	<	>	<	>	-	>
5–6	<	<	<	<	<	<	<	<									-	<	-	<	>	>	>	>	>	>	>	>	<	-	<	<	-	<	<	<
7–8	<	<	<	<	<	<	-	<	-	-	>	-					-	<	>	>	-	<	>	>	<	<	>	-	<	-	<	<	>	-	<	>
9–10	>	-	-	>	>	>	>	>			>	>			>		>	>	>	>	>	>	>	>	>	>	>	>	-	<	-	<	-	>	>	<
11–12	<	<	<	<	<	<	<	<		<							<	<	<	<	<	<	<	<	<	-	<	<	-	-	-	>	>	>	-	-
13–14	-	>	>	>	<	<	<	<	<	<							<	<	<	<	<	-	<	<	<	<	<	<	-	<	>	<	>	>	>	-
15–16	<	<	<	<	-	<	-	<									>	>	>	>	>	>	>	>	>	>	>	-	-	<	<	<	-	>	-	-
17–18	<	<	<	<	-	>	>	>	>								-	>	<	<	-	>	<	<	>	>	<	<	<	-	<	>	-	>	<	-
19–20	>	-	>	-	-	-	>	>	-	<	<	<			<		<	<	<	<	<	-	<	<	<	<	-	-	>	>	>	>	-	>	>	>
21–22	<	<	<	<	-	>	<	-	<	<	<	<			<	<	<	<	<	<	<	<	<	<	<	<	<	<	-	>	>	>	>	>	>	-
23–24	<	<	<	<	-	-	>	>									-	>	<	<	<	>	-	-	>	>	-	-	>	-	>	>	-	-	>	>
25–26	<	<	<	<	<	<	<	<									<	<	<	<	<	<	<	<	<	<	<	<	<	>	>	>	>	<	<	<
27–28	>	>	>	>	-	-	>	-				>					>	>	>	>	>	>	>	>	>	>	>	>	>	<	>	-	>	-	>	<
29–30	<	<	<	<	<	<	<	<									-	-	-	<	<	-	-	<	<	<	<	<	<	<	<	-	>	<	<	<

< parameter greater in uremic than in normal lymph nodes.
- parameter of equal size in both uremic and normal lymph nodes.
> parameter smaller in uremic than in normal lymph nodes.
☐ no comparison made.

A, B two observers.

1, 2 two separate evaluations.

* the pairs were evaluated blind and in a random series.

§ in one lymph node pair (from patient no. 4) both lymph nodes were excised before ECIB, and this patient is therefore only included in consistency evaluations.

Acta path. microbiol. scand. Sect. C, *83:* 298–302, 1975

ROSETTE FORMATION TESTS FOR T AND B LYMPHOCYTES USING FROZEN-STORED CELLS

S. A. Birkeland

Tissue Culture Laboratory, Institute of Pathology, Odense University Hospital, DK-5000 Odense, Denmark

Birkeland, S. A. Rosette formation tests for T and B lymphocytes using frozen-stored cells. Acta path. microbiol. scand. Sect. C, *83:* 298–302, 1975.

The purpose of this study was to establish whether rosette formation tests could be used on frozen-stored lymphocytes and whether the measured ratio of T and B lymphocytes was affected by frozen storage. The number of B cells was evaluated by testing for HEAC-rosettes using *human A-group erythrocytes, rabbit anti-A and mouse complement.* The number of T cells was found by forming E rosettes using sheep red blood cells and absorbed human AB serum. Fresh lymphocytes, frozen-stored lymphocytes and lymphocytes stored for 24 hours in medium TC-199 were tested for rosette formation. For B rosette formation, there was no significant difference between frozen-stored and fresh cells, whereas medium-stored and fresh cells were significantly different. For T rosette formation there were no significant differences between any of the methods used. Thus it seems reasonable to conclude that 1) the differentiation of a lymphocyte *population into T and B lymphocytes is possible in samples which have been frozen-stored* and 2) the method used for frozen-storage does not affect the ratio of T and B lymphocytes.

Key words: T and B lymphocytes; rosette formation; frozen-stored cells

S. A. Birkeland, Kloverɴænget 26 C, DK-5000 Odense, Denmark.

Received 8.i.75 Accepted 18.iii.75

Using rosette formation tests it is possible to differentiate between T and B lymphocytes in a lymphocyte suspension.

Certain lymphocytes are capable of binding *in vitro* to sheep erythrocytes (SRBCs) *forming rosettes (so-called E rosettes).* Although the immunological basis of this binding is not fully understood, a series of studies (*Jondal et al.* 1972; *Wybran et al.* 1971, 1972 and 1973) show that these lymphocytes are of the thymus dependent type. In suitable conditions up to 80 per cent of lymphocytes react in this way.

Other lymphocytes (B lymphocytes) have receptors for antigen-antibody complexes and for complement components and in suitable conditions can form rosettes with human erythrocytes coated with antibody and complement (so-called HEAC rosettes). These lymphocytes comprise up to 20 per cent of the total.

The purpose of this study was to establish whether rosette formation tests could be used on frozen-stored lymphocytes and whether the measured ratio of T and B lymphocytes was affected by frozen storage.

MATERIALS AND METHODS

Blood samples were taken from 45 hospital employees (13 men and 32 women, age range 22–60 years). Heparin (heparin Sodium Novo, USP, 50 I.U./ml) was used as anticoagulant. 4 ml blood was mixed with 4 ml medium TC-199 with Hepes buffer (Flow) with added heparin (60 I.U./ml) and penicillin-streptomycin (Difco; 100 I.U./100 µg per ml respectively). Iron carbonyl powder (0.1 g/ml) and a few glass beads were then added to the mixture and the samples rotated slowly for 20 min at 37° C. Phagocytes with ingested iron were removed with a magnet and lymphocytes isolated using the Ficoll-Isopaque method (centrifugation for 30 min at 2000 rev/min (560 × *g*). The lymphocyte layer in the intermediate zone was pipetted out and washed twice for 10 minutes at 2000 rev/min (560 × *g*) with TC-199 with the additives described above together with 20 per cent pooled human AB serum screened for antibodies.

The lymphocyte suspensions were mixed in the ratio 1:10 with a freezing medium consisting of 50 per cent TC-199 medium with additives, 40 per cent AB serum and 10 per cent dimethylsulfoxide (DMSO) as cryoprotective agent (in one experimental procedure freezing medium without AB serum was used as described below).

A cryobiological freezing apparatus (Cryoson) was used. Immediately after mixing, the freezing programme was started with 1–2° C temperature decrease per minute to approx. –20° C and then 5–6° C/min to approx. –85° C using programmed injections of liquid nitrogen into the freezing chamber. In this way the cell suspensions were brought through the critical phase-transition stage at about –10° C without the destructive effects of intracellular ice-crystal formation. The cells were then stored in an electric deep-freezer at –95° C.

The frozen suspensions were removed from the deep-freezer immediately before use and placed in a 37° C water bath for two to three minutes. They were removed from the water bath immediately before the last ice in the tubes melted, and the cell suspensions transferred to ice-cold centrifuge tubes containing TC-199 with added serum (or without serum as described below). The mixtures were immediately washed twice for six minutes at 1500 rev/min (135 × *g*). The cell suspensions were then ready for the experiments with rosette formation. Further details of the procedures have been described earlier (*Birkeland*, ref. 2). The suspensions obtained were adjusted to 3 × 10^7 lymphocytes per ml.

HEAC Rosettes

Human A-group erythrocytes, rabbit anti-A and mouse complement were used.

Erythrocytes were washed three times with Hanks solution and a 2.5 per cent v/v suspension prepared (100 µl erythrocytes diluted with 4 ml Hanks solution). 1 ml of this suspension was incubated for 30 min at 37° C with 1 ml rabbit-anti-A (State Serum Institute, Copenhagen), prediluted 1:2500 with Hanks solution (10 µl rabbit-anti-A diluted with 25 ml Hanks solution) and then incubated for 30 min at 37° C with 100 µl mouse serum (fresh frozen and stored at –20° C). 3 ml Hanks solution was added and the suspension gently mixed.

Rosettes were formed by mixing 100 µl lymphocyte suspension and 100 µl sensitised erythrocyte suspension. The mixture was centrifuged for 5 min at 500 rev/min (40 × *g*), incubated for 5 min at room temperature and then gently mixed. One drop was transferred to a counting chamber and the number of rosettes formed by 200 lymphocytes recorded.

E Rosettes

Absorbed AB-serum: A volume of SRBCs was washed three times with Hanks solution and 5 ml packed SRBCs incubated for 30 min at 37° C with an equal volume of pooled human AB serum. The cells were removed completely from the serum by five centrifugations (2000 rev/min (560 × *g*), 5 min) and the serum stored at –20° C.

The test: SRBCs stored at 4° C in ACD were washed three times with Hanks solution and an 0.5 per cent v/v suspension prepared (20 µl packed SRBCs diluted with 4 ml Hanks solution). 100 µl of this suspension were incubated for 30 min at 37° C with 100 µl lymphocyte suspension and 20 µl absorbed AB serum. The mixture was centrifuged for 5 min at 500 rev/min (40 × *g*) at room temperature and incubated for 18 hours at 4° C. After gentle mixing, one drop was carefully transferred to a counting chamber and the number of rosettes formed by 200 lymphocytes recorded. Lymphocytes with ≥ 3, 2 and 1 erythrocyte per rosette were counted separately.

Experimental Procedures

Three different kinds of experiments were performed:

I) Comparison of the influence of storage after freezing routinely but without the AB-serum usually added to the freezing medium, and storage in medium TC-199 with added Hepes buffer, heparin and antibiotics.

The lymphocyte suspension was divided in two portions. Portion 1 was frozen immediately in 10 per cent DMSO freezing medium and portion 2 was stored for 24 hours in TC-199. After thawing of portion 1 the two portions were tested for the two types of rosette formation in the same routine run.

II) Comparison of the influence of frozen stor-

TABLE 1. *Comparison Between Frozen-stored and Medium-stored Cells—without and with AB-serum*

Without AB-serum

Patient number	HEAC rosettes: frozen-stored	HEAC rosettes: medium-stored	E rosettes frozen-stored ≥3	2	1	E rosettes medium-stored ≥3	2	1
1	9	15	61	5	2	66	4	2
2	15	17	57	3	9	70	1	9
3	13	16	49	7	5	55	8	6
4	8	12	65	5	7	62	6	2
5	13	19	75	3	2	74	1	2
6	13	17	65	1	6	60	3	8
7	10	12	64	7	1	63	3	3
8	12	16	61	4	3	67	6	1
9	10	14	39	7	14	44	4	18
10	12	13	63	4	7	64	8	6
11	8	16	73	5	0	73	5	1
12	9	20	66	3	3	65	1	3
13	13	15	69	5	1	72	3	1
Mean	11	16	62			64		
± SD	2	2	10			8		

With AB-serum

Patient number	HEAC rosettes: frozen-stored	HEAC rosettes: medium-stored	E rosettes frozen-stored ≥3	2	1	E rosettes medium-stored ≥3	2	1
14	12	18	51	7	15	49	7	18
15	13	14	65	2	2	59	2	4
16	15	13	71	2	1	69	3	3
17	14	19	64	4	5	59	6	7
18	9	13	70	5	2	73	4	1
19	14	19	71	1	1	69	1	3
20	16	15	56	5	3	60	5	7
21	15	18	72	2	2	79	1	3
Mean	14	16	65			65		
± SD	2	3	8			10		

Figures in per cent of lymphocytes examined.

age with AB serum added to the freezing medium and storage in medium TC-199 with added Hepes buffer, heparin and antibiotics.

The freezing medium contained 10 per cent DMSO and 40 per cent AB-serum. Apart from this the experimental procedure was the same as in experiment I.

III) Comparison of fresh lymphocytes, frozen-stored and medium-stored lymphocytes.

The lymphocyte suspension was divided into three portions. Portion 1 was examined immediately together with portion 2, which was frozen immediately (10 per cent DMSO and 40 per cent AB serum in the freezing medium) and then thawed. Portion 3 was stored for 24 hours in medium TC-199 with the usual agents added.

RESULTS

The results of experiments I and II are shown in Table 1. 200 lymphocytes were examined in each case and the results are expressed as percentages of cells examined. The results were evaluated statistically using two column analysis of variance.

For B lymphocytes there were significant differences between the results after the different types of storage used in experiment I ($p < 0.001$) and experiment II ($p < 0.05$). For T lymphocytes there were no significant differences between the methods used.

The results of experiment III, the most important part of the study, are shown in Table 2. The results are given in percentages as in Table 1. The statistical methods used were three columns analysis of variance and Mann Whitney probability test.

24 experiments were carried out in three different trials.

For T rosettes there were no significant differences between any of the methods used.

For B rosettes there was no statistically significant difference in the comparison of frozen-stored and fresh cells, whereas the comparison of medium-stored and fresh cells gave significantly different results ($p < 0.01$).

Average results for B and T lymphocytes are shown in the tables. Rosettes were formed by 82–89 per cent of lymphocytes counted, cells not forming rosettes ("nil-cells") thus accounting for 11–18 per cent of the total counted.

TABLE 2. *Comparison Between Fresh, Frozen-stored and Medium-stored Lymphocytes*

Patient number	HEAC rosettes fresh cells	HEAC rosettes frozen-stored cells	HEAC rosettes medium-stored cells	E rosettes fresh cells ≥3	2	1	E rosettes frozen-stored cells ≥3	2	1	E rosettes medium-stored cells ≥3	2	1
22	20	19	18	67	2	0	59	3	6	60	2	2
23	17	15	15	60	3	7	63	1	0	58	2	3
24	14	13	14	71	2	1	60	1	15	75	3	1
25	14	17	16	66	3	1	69	2	1	62	4	4
26	16	18	19	60	8	3	64	4	3	68	0	3
27	17	19	22	68	0	5	72	1	0	74	3	2
28	15	19	19	74	3	0	77	4	0	67	2	1
29	19	18	17	64	0	2	62	0	2	58	0	4
mean	17	17	18	66			66			65		
± SD	2	2	3	5			6			7		
30	12	13	10	76	0	2	74	0	0	68	0	3
31	14	15	12	75	0	0	72	3	2	64	0	3
32	17	16	13	73	1	0	67	4	1	63	3	2
33	19	18	14	61	6	4	63	2	3	57	5	4
34	17	18	11	70	3	1	69	3	0	70	1	0
35	18	20	17	71	0	10	71	0	8	64	3	8
36	21	22	16	86	0	0	80	0	3	76	0	0
37	21	21	18	64	0	18	69	1	13	65	1	6
mean	17	18	14	72			71			66		
± SD	3	3	3	8			5			6		
38	20	19	13	60	0	4	61	0	3	66	1	0
39	21	18	13	64	1	8	64	0	5	68	0	2
40	17	15	11	72	3	2	70	1	0	71	0	0
41	18	16	16	81	1	1	78	2	0	71	0	0
42	19	18	14	61	1	0	61	1	0	64	0	2
43	18	16	16	61	0	1	58	2	0	64	1	0
44	16	16	18	59	0	19	55	1	12	63	1	0
45	19	20	14	55	2	11	56	1	10	60	0	8
mean	19	17	14	64			63			66		
± SD	2	2	2	8			4			4		
grand mean	17	17	15	67			66			66		
± SD	2	2	3	8			7			5		

Figures in per cent of lymphocytes examined.

DISCUSSION

The three methods compared (fresh, frozen-stored and medium-stored cells) are commonly used for the investigation of lymphocytes in immunological laboratories.

The method for freezing lymphocytes used in experiment III is the routine method in this laboratory. On the basis of this study it seems reasonable to conclude that 1) the differentiation of a lymphocyte population into T and B lymphocytes is possible in samples which have been frozen-stored and 2) the method used for the frozen storage does not affect the ratio of T and B lymphocytes.

By freezing prepared lymphocyte suspen-

sions and collecting series of samples for rosette formation tests, laboratory capacity can be utilised in a rational way. In addition, since all the samples are examined in a single routine session, there are smaller variations in the results of such tests.

"Nil cells", which are a feature of all investigations of rosette formation, could either be T and B lymphocytes with a surface concentration of receptors for rosette formation which is too low to provide a reaction, or they could be representative of independent subpopulations.

Allowing for differences due to modifications of methods, the results presented in this study compare well those of earlier studies. *Mendes et al.* 1973, found 55.4 ± 9.4 per cent E rosettes and 14.4 ± 4.9 per cent HEAC rosettes. *Bentwich et al.* 1973, found 77.1 ± 5.5 per cent E rosettes, while *Jondal et al.* 1972, found 52–81 per cent E rosettes and 25–33 per cent HEAC rosettes.

Thus rosette tests in connection with the investigation of immunological conditions during transplantation or the course of long-term treatment can advantageously be performed on lymphocytes collected using a system of frozen storage.

I am very grateful to *A. Svejgaard*, M.D., Tissue Typing Laboratory and Blood Grouping Department, State University Hospital, Copenhagen, in whose laboratory I learned the rosette formation tests used.

This study was supported by grants from *King Christian X Fund, Ingemann O. Buck's Fund, P. Carl Petersen's Fund, Engineer Soren Alfred Andersen's Fund, C. C. Klestrup and Wife's Legacy* and the *State Medical Research Fund.*

REFERENCES

1. *Bentwich, Z., Douglas, S. D., Siegal, F. P. & Kunkel, H. G.:* Human lymphocyte-sheep erythrocyte rosette formation: some characteristics of the interaction. Clin. Immun. & Immun. path. *1:* 511–522, 1973.
2. *Birkeland, S. A:* Immunological capacity of peripheral lymphocytes in a blasttransformation system using frozen-stored cells. Submitted to Cryobiology.
3. *Jondal, M., Holm, G. & Wigzell, H.:* Surface markers on human T and B lymphocytes I. J. Exp. Med. *136:* 207–215, 1972.
4. *Mendes, N. F., Tolnai, M. E. A., Silveira, N. P. A., Gilbertsen, R. B. & Metzgar, R. S:* Technical aspects of the rosette tests used to detect human complement receptor (B) and sheep erythrocyte-binding (T) lymphocytes. J. Immunol. *111:* 860–867, 1973.
5. *Wybran, J. & Fudenberg, H. H.:* Rosette formation, a test for cellular immunity. Trans. Assoc. Am. Physicians *84:* 239–247, 1971.
6. *Wybran, J., Carr, M. C. & Fudenberg, H. H.:* The human rosette-forming cell as a marker of a population of thymus-derived cells J. Clin. Invest. *51:* 2537–2543, 1972.
7. *Wybran, J. & Fudenberg, H. H.:* Thymus-derived rosette-forming cells. New Engl. J. Med. *288:* 1072–1073, 1973.

Acta path. microbiol. scand. Sect. C, *83:* 303–309, 1975

ANTIBODY RESPONSE TO HAPTEN-PROTEIN CONJUGATES IN CARRIER PRIMED MICE

Heikki Sarvas

Department of Serology and Bacteriology, University of Helsinki, Helsinki, Finland

Sarvas, H. Antibody response to hapten-protein conjugates in carrier primed mice. Acta path. microbiol. scand. Sect. C, *83:* 303–309, 1975.

Priming mice with a protein (bovine serum albumin or ovalbumin) prepared them for a markedly enhanced anti-hapten response to the conjugate of a hapten NIP (4-hydroxy-3-iodo-5-nitrophenacetyl) and the protein. The enhancing effect of this preimmunization was carrier-specific and dependent on the priming dose of the carrier. Thus, on day 6, anti-NIP titers were 6 to 240 times ($p<0.01$–<0.001) higher in groups primed with 0.01 to 10 mg of BSA and challenged with NIP-BSA than in groups not primed with the carrier. The optimum dose (0.1 to 1 mg) of BSA for an enhanced anti-NIP response also resulted in the highest secondary anti-BSA titers. The enhancing effect of the carrier priming was observed only at the beginning of the anti-NIP response. Corresponding findings were obtained when ovalbumin was used for preimmunization and as the carrier in the challenge. This dose-response relationship in mice was different from that found earlier in chickens. Small doses of carrier induced optimal priming in chickens. Priming with higher doses which induced high titers of anti-carrier antibody had a suppressive effect on the anti-hapten response in these birds.

Key words: Antibody response; hapten-protein conjugates; carrier primed mice.

H. Sarvas, Department of Serology and Bacteriology, University of Helsinki, Haartmaninkatu 3, 00290 Helsinki 29, Finland.

Received 28.ii.75 Accepted 28.ii.75

Production of anti-hapten antibody to many hapten-protein conjugates seems to involve an interaction between carrier-specific thymus-derived (T) cells and hapten-specific bone marrow- or bursa-derived (B) cells (24, 25, 28, 29, 32). Prior immunity to the carrier protein may modify the subsequent primary anti-hapten response to the hapten-carrier conjugate (17); enhanced or accelerated anti-hapten responses may be due to the increased amount or capacity of carrier-specific T helper cells (8, 15, 16, 18, 19, 30) whereas decreased anti-hapten responses may be the result of anti-carrier antibodies (10, 13, 14, 30) and/or carrier-specific suppressor T cells (5, 7, 16, 26, 27). The regulatory influence of carrier priming (carrier-specific T cells) has been shown on IgM, IgG and IgE anti-hapten antibodies (12, 15, 19, 31) as well as on the distribution of guinea pig IgG_1 and IgG_2 isotypes in the anti-hapten response (21).

In a previous work with chickens (30) we demonstrated that carrier-priming had a dual effect on the subsequent anti-hapten response. If carrier-priming caused a good humoral anti-carrier response the subsequent anti-

hapten response was reduced while an enhanced anti-hapten response was obtained if carrier-priming did not lead to a considerable anti-carrier formation. A negative carrier-priming could be converted to a positive one not if the carrier-primed chicken itself was challenged but if its lymphoid cells were transferred to cyclophosphamide treated recipients and these recipients were challenged.

This work was undertaken to study the effect of carrier preimmunization in mice. Mice were primed with various doses of a carrier protein and then challenged with the hapten-protein conjugate. Subsequent anti-hapten and anti-carrier titers were determined and the relationship of carrier protein priming dose to these titers was evaluated.

MATERIALS AND METHODS

Antigens. Bovine serum albumin (BSA, fraction V, Armour Pharmaceutical Company Ltd., England) and ovalbumin (OA, grade V, Sigma Chemical Company, St. Louis, Mo., USA) were used as carrier-proteins.

The hapten NIP-cap (4-hydroxy-3-iodo-5-nitrophenacetyl-Nε-aminocaproic acid) was coupled to BSA and OA as follows: N-hydroxysuccinimide ester of NIP-cap (NIP-cap-Su), kindly prepared by Dr. *M. Becker* (1), was dissolved in dioxan to make a 5 per cent solution. Four ml 5 per cent NIP-cap-Su was added to 70 ml of an aqueous solution (2.6 per cent sodium bicarbonate, 15 per cent tertiary butanol) containing 3 g BSA; 0.1 ml 5 per cent NIP-cap-Su was added to 6.5 ml of a solution containing 25 mg OA in 3 per cent sodium bicarbonate. The mixtures were incubated overnight at +4° C, then dialyzed against 3 per cent sodium bicarbonate (several changes) and finally against saline The amount of conjugated hapten was estimated spectroscopically, assuming that at pH 8.9 and 430 nm in a 1 cm cuvette, 10^{-4} M NIP had an absorbance of 0.49 (2). A molecular weight of 67,000 was assumed for BSA and 45,000 for OA. NIP-cap-BSA and NIP-cap-OA both contained 6 moles of NIP-cap per mole of protein. The chloramine-T method (11) was used for the radioiodination of BSA and OA with ^{125}I (NaI, Amersham, England). N^{125}IP-cap was prepared as described previously (2).

Animals and immunizations. (CBA × C57)F_1 mice, 3 to 4 months old, were used throughout. They were preimmunized subcutaneously (four sites) either with carrier-protein emulsified in complete Freund's adjuvant (CFA, Difco Laboratories, Detroit, Mich., USA) in doses shown in Tables 1 and 2 or with saline in CFA. One week later all the mice were injected intraperitoneally with 100 µg of alum-precipitated NIP-BSA or NIP-OA plus 10^9 killed *Bordetella pertussis* bacteria. For the alum-precipitate, one part 0.1 per cent hapten-protein conjugate in saline was mixed with one part 10 per cent $KAl(SO_4)_2 \cdot 12H_2O$ in distilled water, pH raised to 6.5 with 5 N NaOH, and the resulting precipitate washed twice with saline (4). The mice were bled from the tail 6 and 20 days after the conjugate injection. The sera were stored at −20° C until tested.

Antibody assays. Inactivation of NIP-cap-T4 bacteriophage (HPI-test) (1, 22) and the Farr assay (6) as modified by *Brownstone et al* (2, 3) were used to measure anti-NIP antibodies. The modified Farr assay (2, 3) was used to determine anti-BSA and anti-OA antibodies.

RESULTS

Mice primed with BSA and challenged with NIP-BSA tended to have higher titers of anti-NIP than non-primed control mice (Table 1). Thus, on day 6, anti-NIP titers were 7.6 to 240 times ($p<0.01$–<0.001) higher in groups primed with 0.01 to 1 mg of BSA than in groups not primed with the carrier. When 10 mg of BSA was used for priming the effect of preimmunization on anti-NIP was decreased (Table 1). The optimum dose (0.1 to 1 mg) of BSA for an enhanced anti-NIP response also resulted in the highest secondary anti-BSA titers. When 0.1 or 1.0 µg of BSA was used for preimmunization there was no effect on day 6 anti-NIP titers. Nor did these doses prime for detectable secondary anti-BSA production (Table 1).

The effect of carrier priming on day 20 anti-NIP titers varied: In some experiments the enhancing effect was still seen with 0.01, 0.1 and 1 mg doses of BSA, whereas in one experiment the anti-NIP titers in carrier primed groups were equal or lower than in the control mice (Table 1). The effect of carrier priming on day 20 anti-BSA titers also varied: Enhanced anti-BSA titers were seen in groups primed with 1 or 10 mg of BSA, while priming with 0.01 or 1.0 mg of BSA had either an enhancing or zero effect on anti-BSA titers (Table 1). The highest

TABLE 1. *Effect of Preimmunization with Bovine Serum Albumin (BSA) on the Primary Anti-NIP Response to NIP-BSA in Mice*[a)]

	of mice	Priming dose of BSA in CFA on day –7 (µg/mouse)	Antibodies on day 6		Antibodies on day 20		
			Anti-NIP HPI[b)]	Anti-BSA[c)]	Anti-NIP HPI	Anti-NIP HBC[d)]	Anti-BSA
I	10	100	2,600,000***	28*	23,000,000	67	88***
			°	°°°		°°	°°
	9	10	920,000***	<1.1	40,000,000**	160**	23*
			°°°		°°	°°	°
	9	1	15,000	<0.20	9,600,000	37	3.2
	9	0.1	14,000	<0.23	n.d.	75	4.1
	10	–	11,000	<0.20	14,000,000	76	13
II	10	10,000	190,000***[e)]	1.1	n.d.	2.5***	33**
			°			°	
	8	1,000	850,000***	4.7*	n.d.	11**	41***
	10	100	930,000***	4.0*	n.d.	39	10
			°°°	°°°			
	9	10	41,000**	<0.20	n.d.	81	6.5
	9	–	5,400	<0.20	n.d.	75	4.8
III	8	10,000	60,000**	3.2*	120,000	0 82	59***
			°°°	°°°	°°°	°°°	°
	8	1,000	1,900,000***	19***	2,700,000***	15***	160***
			°°				°°
	7	100	200,000***	20***	520,000*	3.1*	66***
	5	–	9,600	<0.20	34,000	<0.33	<0.20

a) Mice were preimmunized subcutaneously (on day –7) either with BSA in complete Freund's adjuvant (CFA) or with saline in CFA. One week later (on day 0) all the mice were injected intraperitoneally with 100 µg of alum-precipitated (4-hydroxy-3-iodo-5-nitrophenacetyl) caproic acid coupled to BSA (NIP-BSA) plus 10^9 killed *Bordetella pertussis* bacteria. All the titers are geometric means.

b) HPI = haptenated phage inactivation.

c) Micrograms of BSA bound/ml of serum at the concentration of ca. 1 µg/ml of free BSA.

d) HBC = hapten binding capacity, in 10^{-8} moles of NIP-cap bound/liter of serum at ca. 10^{-8}M concentration of free hapten. If less than 1 per cent of hapten or antigens was bound, the percentage bound was arbitrarily set at 1 per cent.

e) Most of the day 6 sera in experiment II were also measured for anti-NIP by the HPI-test in the presence of 0.1 M 2-mercaptoethanol (2-ME) (20). Data showed that enhanced anti-NIP was mainly 2-ME-sensitive.

Asterisks indicate the confidence of the difference between the carrier-primed and the control (not primed with the carrier-protein) group. Circles between two numbers indicate the confidence of the difference between these two numbers (* or ° $p<0.05$; ** or °° $p<0.01$; *** or °°° $p<0.001$; Student's two tailed t-test). n.d. = not done.

day 20 anti-BSA titers were seen in groups primed with 1 mg of BSA. These groups had either enhanced or reduced anti-NIP titers (Table 1).

When mice were primed with OA and challenged with NIP-OA the findings were similar to those obtained when BSA was used as a carrier. Carrier preimmunization increased the day 6 anti-NIP HPI-titer about 10-fold in mice primed with 2 mg and about 30-fold in mice primed with 0.2 mg of OA (Table 2). The enhancing effect of the priming on anti-NIP response was lost by day 20; the anti-NIP titers of primed mice were equal or lower than those in the controls (Table 2). Both day 6 and day 20 anti-OA titers were significantly ($p<0.001$) enhanced in groups primed with OA. Without carrier preim-

TABLE 2. *Effect of Preimmunization with Ovalbumin (OA) or Bovine Serum Albumin (BSA) on the Primary Anti-NIP Response to NIP-OA in Mice*

No. of mice	Priming dose in CFA on day −7	Challenge on day 0[a]	Antibodies on day 6				Antibodies on day 20		
			Anti-NIP		Anti-OA[b]	Anti-BSA	Anti-NIP HBC	Anti-OA	Anti-BSA
			HPI	HBC					
Exp. I 9	2,000 μg OA	NIP-OA	3,700,000***	5.0*** °°°	95***	n.d.	44*** °°°	454***	n.d.
9	200 μg OA	NIP-OA	11,000,000***	18***	100***	n.d.	290	230***	n.d.
9	–	NIP-OA	340,000	<0.25	<1.0	<0.20	330	<1.3	<0.23
Exp. II 9	10,000 μg BSA	NIP-OA	320,000	<0.43	n.d.	5.2*** °°°	370	n.d.	56*** °°°
9	1,000 μg BSA	NIP-OA	400,000	<0.24	n d.	19***	290	n.d.	250*** °°°
9	100 μg BSA	NIP-OA	230,000	<0.25	n.d.	18***	310	n.d.	51***

[a]) 100 μg of alum-precipitated NIP-OA plus 10^9 *Bordetella pertussis* bacteria were given intraperitoneally.
[b]) Micrograms of OA bound/ml of serum at the concentraton of ca. 5 μg/ml of free OA.
Asterisks indicate the confidence of the difference between the carrier-primed and the control (not primed with the carrier-protein) group. Circles between two numbers indicate the confidence of the difference between these two numbers (* or ° $p<0.05$; ** or °° $p<0.01$; *** or °°° $p<0.001$; Student's two tailed t-test). n.d. = not done For further explanations see Table 1.

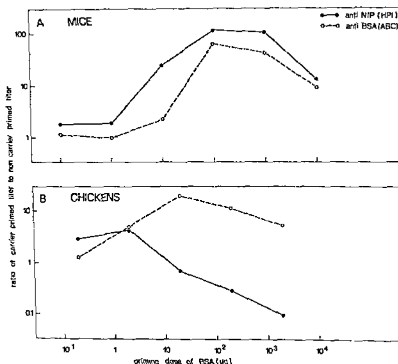


Fig. 1. Carrier priming effect of different doses of bovine serum albumin (BSA) on primary anti-NIP and secondary anti-BSA titers in mice (A) and in chickens (B). The vertical axis indicates the ratio of the geometric mean of carrier primed titers to the geometric mean of non-carrier primed titres. The horizontal axis indicates the doses of BSA used for preimmunization. Both axes are in $\log_{10}$-scale. Each geometric mean was counted from the individual titers of 9–25 mice or 7–13 chickens. Mice and chickens were preimmunized subcutaneously either with BSA in complete Freund's adjuvant (CFA) or with saline in CFA. One week later all the animals were immunized intraperitoneally with alum-precipitated NIP-BSA (100 µg/mouse; 1 mg/chicken) plus killed *Bordetella pertussis* bacteria. Anti-NIP and anti-BSA titers were measured in the sera bled 6 days after the conjugate injection. The mouse data consisted of all the 6-day titers of the experiments I-III (Table 1) of this paper and the chicken data consisted of all the 6-day titers of experiment 1 (Table 3) and experiments 3 and 4 (Table 1) of reference (30). HPI = haptenated phage inactivation. ABC = antigen binding capacity, micrograms of BSA bound/ml of serum at the concentration of ca. 1 µg/ml of free BSA.

munization very little anti-OA could be detected at either bleeding. The priming was carrier-specific; BSA did not modify the subsequent anti-NIP response to NIP-OA (Table 2).

DISCUSSION

The results in this study confirm earlier findings (15, 18, 19) that prior immunity to the carrier-protein enhances or accelerates the early primary anti-hapten response of animals immunized with the hapten-protein conjugate. Furthermore, the enhancing effect of the preimmunization was carrier-specific and dependent on the priming-dose of the carrier-protein.

Small doses of anti-carrier antibody have been shown to enhance (23) and high doses to suppress (13, 14) anti-hapten response to the hapten-carrier conjugate. In the experiments reported here the helper effect seemed to correlate with the secondary anti-carrier response (Fig. 1A), indicating that enhanced primary anti-hapten responses could coexist with large amounts of humoral anti-carrier antibody. This dose-response relationship in mice was quite different from that seen in

chickens (Fig. 1B) (30). Small doses of carrier suboptimal of anti-carrier induction induced optimal priming in chickens. Priming-doses of carrier which induced high titers of anti-carrier antibody reduced the anti-hapten response.

Possible reasons for the different dose-response curves (Fig. 1, A and B) in mice and chickens may be that (i) chicken anti-carrier antibodies compete more efficiently for the hapten-carrier conjugate than mouse antibodies; (ii) blocking antigen-antibody complexes on T cell surfaces (10) or (iii) suppressor T cells (5, 27) are more important in chickens than in mice. The finding (30) that the suppressing effect of the carrier priming after high doses of carrier could be reversed by transfer of primed spleen lymphocytes in chickens may support both (i) and (ii) but probably not (iii) (9).

I wish to thank Prof. O. Mäkelä for valuable discussions and for the bacteriophage NIP-cap-T4 and Dr. Martin Becker for the preparation of the N-hydroxysuccinimide ester of NIP-cap.

REFERENCES

1. *Becker, M. & Mäkelä, O.:* Modification of bacteriophage with hapten-ε-aminocaproyl-N-hydroxysuccinimide esters; increased sensitivity for immunoassay. Immunochemistry, in press.
2. *Brownstone, A., Mitchison, N. A. & Pitt-Rivers, R.:* Chemical and serological studies with an iodine-containing synthetic immunological determinant 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) and related compounds. Immunology *10:* 465–479, 1966
3. *Brownstone, A., Mitchison, N. A. & Pitt-Rivers, R.:* Biological studies with an iodine-containing synthetic immunological determinant 4-hydroxy-3-iodo-5-nitrophenylacetic acid NIP and related compounds. Immunology *10:* 481–492, 1966.
4. *Dunsford, I. & Bowley, C. C.:* Techniques in Blood Grouping. Oliver and Boyd, Edinburgh and London, 1955, p. 134.
5. *Elson, C. J. & Taylor, R. B.:* The suppressive effect of carrier priming on the response to a hapten-carrier conjugate. Eur. J. Immunol. *4:* 682–687, 1974.
6. *Farr, R. S.:* A quantitative immunochemical measure of the primary interaction between I*BSA and antibody. J. infect. Dis. *103:* 239–262, 1958.
7. *Fauci, A. S. & Johnson, J. S.:* Suppression of antibody synthesis. II. The effect of carrier-specific cells upon haptenic competition. J. Immunol. *107:* 1057–1062, 1971.
8. *Fidler, J. M., McDaniel, E. M. & Golub, E. S.:* Regulation of the immune response. III. Effect of the "accelerated" response on hapten-carrier responses. Cell. Immunol. *4:* 29–38, 1972.
9. *Gershon, R. K. & Kondo, K.:* Infectious immunological tolerance. Immunology *21:* 903–914, 1971.
10. *Gorczynski, R., Kontiainen, S., Mitchison, N. A. & Tigelaar, R. E.:* Antigen-antibody complexes as blocking factors on the T lymphocyte surface. In: Edelman, G. M. (Ed.): Cellular Selection and Regulation in the Immune Response, Raven Press, New York 1974, pp. 143–154.
11. *Greenwood, F. C., Hunter, W. M. & Glover, J. S.:* The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem. J. *89:* 114–123, 1963.
12. *Hamaoka, T., Newburger, P. E., Katz, D. H. & Benacerraf, B.:* Hapten-specific IgE antibody responses in mice. III. Establishment of parameters for generation of helper T cell function regulating the primary and secondary responses of IgE and IgG B lymphocytes. J. Immunol *113:* 958–973, 1974.
13. *Hamaoka, T., Takatsu, K. & Kitagawa, M.:* Antibody production in mice. IV. The suppressive effect of anti-hapten and anti-carrier antibodies on the recognition of hapten-carrier conjugate in the secondary response. Immunology *21:* 259–271, 1971.
14. *Haughton, G. & Mäkelä, O.:* Suppression or augmentation of the antihapten response in mice by antibodies of different specificities. J. exp Med. *138:* 103–115, 1973.
15. *Hurme, M., Kontiainen, S., Seppälä, I. J. T. & Mäkelä, O.:* Affinity and Ig classes of antihapten antibodies in carrier-preimmunized rats. Eur. J. Immunol *3* 191–195, 1973.
16. *Ishizaka, K. & Okudaira, H.:* Reaginic antibody formation in the mouse. II. Enhancement and suppression of antihapten antibody formation by priming with carrier. J. Immunol *110:* 1067–1076, 1973.
17. *Katz, D. H. & Benacerraf, B.:* The regulatory influence of activated T cells on B cell responses to antigen. Advanc. Immunol. *15:* 1–94, 1972.
18. *Katz, D. H., Paul, W. E., Goidl, E. A. & Benacerraf, B.:* Carrier function in anti-hapten immune responses. I. Enhancement of primary and secondary anti-hapten antibody responses

by carrier preimmunizatios. J. exp. Med. *132:* 261–282, 1970.

19. *Kontiainen, S.:* The effect of carrier-primed cells on an adoptive anti-hapten response. Eur. J. Immunol. *1:* 276–280, 1971.
20. Kontiainen, S. & Mäkelä, O.: Determination of 19S and 7S components in an anti-hapten antibody . Ann. Med. exp. Fenn. *45:* 472–476, 1967.
21. *Liu, S. H., Koo, P. H. & Cebra, J. J.:* Effect of carrier priming on the distribution of anti-hapten antibodies between IgG1 and IgG2 isotypes in the hyperimmune guinea pig. J. Immunol. *113:* 677–687, 1974.
22. *Mäkelä, O.:* Assay of anti-hapten antibody with the aid of hapten-coupled bacteriophage. Immunology *10:* 81–86, 1966.
23. *McBride, R. A. & Schierman, L. W.:* Passive antibody and the immune response. Factors which determine enhancement and suppression. J. exp. Med. *134:* 833–845, 1971.
24. *Mitchison, N. A.:* The carrier effect in the secondary response to hapten-protein conjugates. II. Cellular cooperation. Eur. J. Immunol. *1:* 18–27, 1971.
25. *Mitchison, N. A., Rajewsky, K. & Taylor, R. B.:* Cooperation of antigenic determinants and of cells in the induction of antibodies. In: Sterzl, J. (Ed.): Developmental aspects of antibody formation and structure, Academic Press, New York 1970, pp. 547–564.
26. *Okumura, K. & Tada, T.:* Regulation of homocytotropic antibody formation in the rat. VI. Inhibitory effect of thymocytes on the homocytotropic antibody response. J. Immunol. *107:* 1682–1689, 1971.
27. *Okumura, K. & Tada, T.:* Suppression of hapten-specific antibody response by carrier-specific T cells. Nature (New Biol.) *245:* 180–182, 1973.
28. *Raff, M. C.:* Role of thymus-derived lymphocytes in the secondary humoral immune response in mice. Nature *226:* 1257–1258, 1970.
29. *Rajewsky, K., Schirrmacher, V., Nase, S. & Jerne, N. K.:* The requirement of more than one antigenic determinant for immunogenicity. J. exp. Med. *129:* 1131–1143, 1969.
30. *Sarvas, H., Mäkelä, O., Toivanen, P. & Toivanen, A.:* Effect of carrier preimmunization on the anti-hapten response in the chicken. Scand. J. Immunol. *3:* 455–460, 1974.
31. *Schirrmacher, V. & Rajewsky, K.:* Determination of antibody class in a system of cooperating antigenic determinants. J. exp. Med. *132:* 1019–1034, 1970.
32. *Weinbaum, F. I., Gilmour, D. G. & Thorbecke, G. J.:* Immunocompetent cells of the chicken. III. Cooperation of carrier sensitized T cells from agammaglobulinemic donors with hapten immune B cells. J. Immunol. *110:* 1434–1436, 1973.

Acta path. microbiol. scand. Sect. C, *83:* 310–314, 1975

INFLUENCE OF METHYLPREDNISOLONE AND AZATHIOPRINE ON POLYMORPHONUCLEAR NEUTROPHILS (PMN) IN RAT PERITONEAL EXUDATES

A. Baardsen

Kaptein Wilhelm Wilhelmsen og Frues Bakteriologiske Institutt and Institute for Surgical Research, University Hospital, Rikshospitalet, Oslo, Norway

Baardsen, A. Influence of methylprednisolone and azathioprine on polymorphonuclear neutrophils (PMN) in rat peritoneal exudates. Acta path. microbiol. scand. Sect. C, *83* 310–314, 1975.

In rats treated parenterally with high doses of methylprednisolone or azathioprine, peritoneal exudates were obtained by i.p. injection of sodium caseinate. Treatment by either drug caused a 50 per cent reduction of the PMN influx as compared to control animals simultaneously studied. The drug action evidently depends upon different mechanisms. Methylprednisolone blocked the emigration of PMN, while the low influx in the azathioprine-treated rats was related to a lowered count of PMN in blood. The volume of exudates was not affected by drug treatment. *The phagocytic activity of peritoneal PMN as expressed by uptake of ^{32}P-labelled E. coli, was found to be intact in either group of drug-treated rats.*

Key words: Methylprednisolone; azathioprine; inflammatory exudates; neutrophils; rats.

A. Baardsen, Kaptein Wilhelm Wilhelmsen og Frues Bakteriologiske Institutt and Institute for Surgical Research, University Hospital, Rikshospitalet, Oslo, Norway.

Received 4.ii.75 Accepted 11.iii.75

The phagocytic capacity of PMN at the site of inflammation is important to the host resistance against invading microorganisms. The number of PMN as well as their phagocytic activity are possible targets of infection-enhancing drugs such as glucocorticosteroids (steroids) and azathioprine (AZA).

As noted by *Boggs & Athens* (3), conflicting results have characterized studies of the effect of steroids upon the cellular composition of inflammatory exudates. This might partly be due to the use of inadequate methods (10). Methods including determination of the volume of exudates would probably give a more correct picture of the cellular influx. PMN reach the site of inflammation from the blood stream. Simultaneous counts of PMN in blood and exudate might yield information about a drug-induced alteration of the influx of these cells related to a certain inflammatory stimulus.

Although AZA is used in auto-immune and chronic inflammatory diseases, its effect upon the influx of PMN into inflammatory exudates has not been thoroughly investigated.

Whether steroids reduce the bactericidal activity of PMN is not definitely settled (5). In a previous study (1) of rat peritoneal PMN, pre-incubation of these cells with methylprednisolone (MP) did not reduce their uptake of ^{32}P-labelled *E. coli in vitro.*

It is possible, hovewer, that protracted *in vivo* exposure of PMN to steroids might influence the cellular uptake.

The biological effect of AZA depends upon *in vivo* conversion to active metabolites (2). *In vitro* studies of the effect of AZA on the function of PMN would therefore not yield information about *in vivo* effect of this drug.

It has been the aim of the present study on rats to evaluate the influence of treatment with MP or AZA on the influx of PMN into peritoneal exudates, as well as on the phagocytic activity of these cells.

MATERIALS AND METHODS

Animals

Four series of 15–18 conventional rats from two different strains, CDF (Charles River Breeding Labs., Wilmington, Mass. U.S.A.) and Wistar, were used. Three of the series were used with regard to sex and one without regard to sex. Each series was divided into 3 groups comprising equal numbers of rats, i.e. one group serving as controls, one group treated with MP and one group treated with AZA.

Drug Treatment

MP in aquous solution ((Solu-Medrol®) The Upjohn Company, Kalamazoo, Mich. U.S.A.) was given s.c. in daily doses of 40 mg per kg for 3 weeks to the MP-treated group of rats.

AZA ((Imuran®) Burroughs Wellcome & Co.) in aquous solution was injected intraperitoneally (i.p.) once daily for 3 weeks. A standard dose of 40 mg per kg per day was used.

Peritoneal Exudates

were induced by i.p. injection of sodium caseinate (12) together with the last drug injection. 21 hours later the exudates were harvested. This procedure was carried out in rats anaesthetized with ether-ethanol 1:1 (v/v). 20 ml of a Krebs-Ringer phosphate buffer with 10 mM glucose (KRG) and 10 I.U. heparin per ml was injected i.p. After gentle massage, the i.p. fluid was aspirated through a 1,2 gauge needle and individual exudate volumes were recorded. To assure complete evacuation, the abdomen was finally opened.

Leucocyte Counting

and Differential Leucocyte Counting

on exudates and blood were carried out as previously described (2).

Determination of Phagocytosis

The phagocytic activity of pooled peritoneal PMN was determined as previously described (12). Using 15 min at 37° C as a standard period of incubation, the ability of glass-adherent PMN to engulf ^{32}P-labelled *E. coli* was measured in the presence of fresh standard serum (5 per cent) from non-treated CDF rats.

Viability

of peritoneal leucocytes was judged by their ability to exclude Trypan blue (5).

Statistical Analysis

was carried out by the two-samples ranks test of Wilcoxon-White (9).

RESULTS

The neutrophilic response to caseinate stimulation is shown in Table 1. The PMN count in blood after caseinate stimulation was doubled in controls ($p<0.01$) compared to PMN blood counts prior to stimulation. In either treated group of rats, the starting-point was different, neutropenia being present in the AZA-treated group and neutro-

TABLE 1. *Influence of i.p. Caseinate Stimulation on the Number of PMN per mm³ Blood in Female CDF Rats Treated with Azathioprine (AZA), Methylprednisolone (MP), and in Controls*

Group	No. of rats	Immediately before PMN ± SEM	21 hours after stimulation PMN ± SEM	Increase PMN ± SEM	Per cent
AZA	6	559 ± 131	799 ± 168	240 ± 211	42.9
MP	5	2324 ± 354	3494 ± 1256	1171 ± 913	50.4
Controls	6	1205 ± 69	2416 ± 343	1211 ± 313	100.5

SEM = standard error of the mean.

TABLE 2. *Influence of Treatment with Azathioprine (AZA) or Methylprednisolone (MP) on the Volume of Peritoneal Exudates and its Content of PMN 21 Hours after i.p. Caseinate Stimulation in Female CDF Rats*

Group	No. of rats	Exudate ml ± SEM	No. of PMN per mm³ ± SEM	Total number of peritoneal PMN ± SEM
AZA	6	17.0 ± 0.6	10838 ± 492	$18 \times 10^7 \pm 0.87 \times 10^7$
MP	5	16.6 ± 0.5	10138 ± 896	$17 \times 10^7 \pm 1.90 \times 10^7$
Controls	6	17.1 ± 0.9	22244 ± 3638	$38 \times 10^7 \pm 6.86 \times 10^7$

SEM = standard error of the mean.

TABLE 3. *Per Cent Trypan Blue Negative Leucocytes in Pooled Peritoneal Exudates Harvested 21 Hours after Caseinate Stimulation of Wistar Rats Treated with Methylprednisolone (MP) or Azathioprine (AZA)*

Group	Male		Female	
	Per cent	No. of rats	Per cent	No. of rats
MP	91	5	98	5
AZA	92	5	92	5
Controls	96	5	98	5

TABLE 4. *Influence of Treatment with AZA or MP on the Ratio* $\frac{PMN/mm^3\ Exudate}{PMN/mm^3\ Blood}$ *(R) in Female CDF Rats 21 Hours after Caseinate Stimulation*

Group	No. of rats	Exudate		Blood		R
		WBC ± SEM	PMN ± SEM	WBC ± SEM	PMN ± SEM	
AZA	6	12990 ± 694	10838 ± 492	3917 ± 805	799 ± 168	16.2 ± 2.9
MP	5	12553 ± 894	10138 ± 896	5240 ± 1259	3494 ± 1256	3.9 ± 0.8
Controls	6	26539 ± 4237	22244 ± 3638	5000 ± 508	2416 ± 343	9.6 ± 1.2

SEM = standard error of the mean. For details see Table 1.

philia in the MP-treated group of rats. The caseinate-induced increase in blood counts was, however, of the same proportion in the two treated groups of rats, 43 per cent in the MP-treated group ($p>0.10$) and 50 per cent in the AZA-treated group ($p>0.10$).

The volume of peritoneal exudates was approximately 17 ml (aspirated volume-20 ml KRG injected), as shown in Table 2. No significant difference ($p>0.10$) between controls and either treated group of rats was found.

The percentage of pooled peritoneal leucocytes excluding trypan blue is shown in Table 3. In all groups, more than 90 per cent of the whole leucocyte population (including PMN) was found to be trypan blue negative.

The number of PMN per mm³ exudate of MP-treated and AZA-treated rats (Table 2) was only 50 per cent of that found in controls ($p<0.01$). Using constant exudate volume, it follows that the total number of PMN present in the peritoneal cavity varied proportionally with the PMN count.

The ratio $\frac{PMN/mm^3\ exudate}{PMN/mm^3\ blood}$ (R) showed a marked difference ($p<0.01$) between con-

TABLE 5. *Uptake of ³²P-labelled* E. coli *by Peritoneal PMN from Rats Treated with Methylprednisolone (MP) or Azathioprine (AZA)*

Group	CDF	Wistar
MP	104.0 ± 26.7 n = 4	104.4 ± 14.4 n = 8
AZA	116.7 ± 7.8 n = 4	106.5 ± 12.1 n = 8
Controls	100.0 ± 9.2 n = 4	100.0 ± 13.2 n = 7

Serum from non-treated rats was used to opsonize. The figures are given in per cent ± 1 standard deviation. The mean uptake rate per mg cell protein of PMN from controls is set as 100 per cent. n = Number of observations, quadruplets per experiment. In CDF rats n is based on 1 series of animals, and in Wistar rats n is based on 2 series. Pooled PMN from 5 rats were used in each group.

trols and the MP-treated group. R was approximately 10 incontrols and 4 in the MP-group (Table 4). In the AZA-group R was 16, $p = 0.10$ compared to controls.

The rate of uptake of ³²P-labelled *E. coli* by pooled peritoneal PMN is shown in Table 5. No significant difference ($p > 0.10$) between controls and either treated group of rats was found.

DISCUSSION

The results of the present investigation show that the quantity of the "late" exudate 21 hours after stimulation was remarkably constant from one group of rats to the other. This observation may support the suggestion by *Hurley* (6) that the vascular permeability has returned to normal before the "late or delayed" PMN emigration begins.

The present method of aspiration enabled calculation of the total number of PMN present in the peritoneal cavity, i.e. 3.82×10^9 in control rats. The total turnover of PMN in these rats is not known. Presuming analogy between rat and man (with PMN turnover 1.26×10^{11} in a 70-kg man per day (11)), a calculation on weight basis might indicate that 70 per cent of the daily turnover of PMN in the present control rats accumulated in the peritoneal exudate. The present findings seem to indicate that a considerable portion of the total PMN pool was drawn to the inflamed peritoneum.

The influx of PMN was reduced to 50 per cent in rats treated with either MP or AZA. As indicated by the ratio $\frac{\text{PMN/mm}^3 \text{ exudate}}{\text{PMN/mm}^3 \text{ blood}}$ (R), the mechanism behind the reduced influx was different in the two groups. In the case of AZA, R was maintained and even increased. In other words, there was no drug-induced inhibition of the forces responsible for the cellular accumulation. It seems likely, therefore, that the low influx of PMN in this case was directly related to the low blood count (2).

The lowered R in MP-treated rats indicates that emigration from the blood vessels was blocked in spite of a high blood count of PMN. From the present investigation it is not possible to relate the action of steroids specifically to production and release from the bone marrow, changes in the local microcirculation, margination of leucocytes, adherence to endothelium, permeability changes of the latter, diapedesis into the extravascular compartment, or directional movement in response to chemostatic gradients. With regard to the influence of steroids on PMN chemotaxis, conflicting results have been reported (4, 7, 13, 14). Whether PMN adherence to endothelium is impaired by steroids *in vivo* is unknown, although it has been observed *in vitro* that prednisone inhibits the adherence of human PMN to nylon fibres (8).

The viability of the peritoneal leucocyte population did not appear to be reduced by treatment with either MP or AZA. The 5 per cent variation found by the trypan blue exclusion test, is not considered to exceed the variation in differential counts of these cells.

The phagocytic activity of peritoneal PMN was not found to be impaired following administration of MP or AZA at high dose

levels. However, the fact that these drugs markedly reduced the number of PMN in exudates, probably means that the defence against invading microorganisms is correspondingly reduced.

REFERENCES

1. *Baardsen, A., Trippestad, A. & Midtvedt, T.: In vitro* influence of glucocorticosteroids on opsonic and bactericidal activities against *Escherichia coli* in rat serum. Acta path. microbiol. scand. Sect. B, *82:* 696–700, 1974.
2. *Baardsen, A., Midtvedt, T. & Trippestad, A.:* Influence of methylprednisolone and azathioprine on polymorphonuclear neutrophils (PMN) and lymphocytes in germfree, monocontaminated and conventional rats. Acta path. microbiol. scand. Sect. C, *83:* 210–214, 1975.
3. *Boggs, D. R. & Athens, J. W.:* The effect of adrenal glucocorticosteroids upon the cellular composition of inflammatory exudates. Amer. J. Pathol. *44:* 763–773, 1964.
4. *Borel, J. F.:* Effect of some drugs on the chemotaxis of rabbit neutrophils *in vitro.* Experientia *29:* 676–678, 1973.
5. *van Furth, R. & van Zwet, T. L.: In vitro* determination of phagocytosis and intracellular killing by polymorphonuclear and mononuclear phagocytes. In: Wein, D. M. (Ed.): Handbook of Experimental Immunology, Sec. Ed. Vol. 2, Blackwell Sc. Publ. Oxford/London/Edinburgh, 1973, p. 36.1–36.23.
6. *Hurley, J. V.:* Substances promoting leucocyte emigration. Ann. NY Acad. Sci. *116:* 918–935, 1964.
7. *Keller, H. U. & Sorkin, E.:* Chemotaxis of leucocytes. Experientia *24:* 641–652, 1968
8. *Mac Gregor, R. R., Spagnuolo, Ph. J. & Letnek, A. L.:* Inhibition of granulocyte adherence by ethanol, prednisone and aspirin, measured with an assay system. New Engl. J. Med. Medical Progress, pp. 642–646, September 1974.
9. *Mainland, D.:* Elementary medical statistics. 2. ed. W. B. Saunders Company, Philadelphia and London, 1964.
10. *Senn, H., Holland, J. F. & Banerjee, T.:* Kinetic and comparative studies on localized leucocyte mobilization in normal man. J. Lab. Clin. Med. *74:* 742–756, 1969.
11. *Stossel, Th. P.:* Phagocytosis (First of three parts). New Engl. J. Med Medical Progress, pp. 717–723, March 1974.
12. *Trippestd, A. & Midtvedt, T.:* Phagocytosis of ^{32}P-labelled *E. coli* by rat peritoneal polymorphonuclear leucocytes Evaluation of a method. Acta path. microbiol. scand. *74:* 259–273, 1968.
13. *Ward, P. A* · Natural and synthetic inhibitors of leucotaxis. In: Lepow, I. H. & Ward, P A. (Eds.): *Inflammation Mechanisms* and control. Academic Press, New York 1972, pp. *301–307.*
14. *Ward, P. A.:* The chemosuppression of chemotaxis. J. Exp. Med. *124* · 209–226, 1966

Acta path. microbiol. scand. Sect. C, *83:* 315–324, 1975

COMPLEMENT FIXATION WITH PORCINE SERUM ANTIBODIES

Enhancing Effect of Porcine C1q on Complement Fixation with IgG Prepared from Porcine Pseudorabies Immune Serum

Morten Eskildsen

The State Veterinary Institute for Virus Research, Lindholm, Kalvehave, Denmark

Eskildsen, M. Complement fixation with porcine serum antibodies. Enhancing effect of porcine C1q on complement fixation with IgG prepared from porcine pseudorabies immune serum. Acta path. microbiol. scand. Sect. C, *83:* 315–324, 1975.

The complement fixing capacity of porcine IgG-antibodies prepared from pseudorabies immune sera was examined in the presence and absence of preparations containing porcine C1q. Preparation of porcine C1q was performed by initial dialysis of normal swine serum against a 0.018 M EGTA buffer, pH 7.5, followed by purification on Sephadex G-200 and subsequently on QAE-Sephadex. The C1q activity in the different preparations was determined by haemolytic assay. Guinea pig complement depleted of C1q by dialysis in 0.018 M EGTA buffer was used as R-C1q. C1 activity was assayed, using guinea pig complement diluted in a Na_2Mg-EDTA buffer as R-C1. The purity of the C1q preparations was estimated by crossed immunoelectrophoresis and rocket immunoelectrophoresis, and it was found that porcine C1q of reasonable purity was obtained by the QAE-Sephadex separation. Whether a microtechnique using four units of haemolytic complement (100 per cent) or a quantitative technique using fifty units of haemolytic complement (50 per cent) were applied, porcine C1q was found to be of crucial importance for complement fixation with porcine antibody(IgG)-antigen complexes. This points to a selective requirement for porcine C1q as opposed to guinea pig C1q.

Key words: Complement fixation; porcine C1q; porcine pseudorabies immune serum; IgG.

M. Eskildsen, The State Veterinary Institute for Virus Research, Lindholm, DK-4771 Kalvehave, Denmark.

Received 31.iii.75 Accepted 31.iii.75

Demonstration of specific antibodies in swine immune serum by direct complement fixation is generally associated with rather unsatisfactory results. Especially at low dilutions, swine serum exhibits a pronounced procomplementary activity which may mask the presence of specific complement fixation (2, 4, 7, 9, 20). Furthermore, the usual heat-treatment of immune serum before use seems to destroy most of a factor (or factors) which is of great importance for the specific fixation of complement with antibodies of porcine origin. This latter difficulty has been overcome by the addition to the complement of normal calf serum not exposed to heat treatment (8, 5, 6) or normal swine serum not exposed to heat treatment (18). In experiments with bovine immune sera, *Knight* & *Cowan* (11) emphasized similarities between C1 and the complement-fixing enhancing material in normal calf serum not exposed to heat treatment.

The present investigation is based on the

20*

observation that porcine pseudorabies immune sera lost completely the ability to fix guinea pig complement after dialysis at low ionic strength in the presence of a chelating agent, followed by removal of a fine precipitate by high speed centrifugation. According to *Yonemasu* & *Stroud* (23), C1q can be precipitated completely from human serum under these conditions.

The aim of the present study was to examine the complement fixation of porcine IgG-antibodies and the importance of the presence of preparations containing porcine C1q.

MATERIALS AND METHODS

Preparations containing porcine C1q. The source of porcine C1q was a pool of sera from adult, specifically pathogen-free (SPF) pigs. a) The C1q was precipitated by dialysis at 4° C of 50 ml swine serum against 500 ml 0.018 M buffer of Ethyleneglycol bis (beta-amino-ethyl)N,N'-Tetra-Acetic acid (EGTA) (E 3251 from Sigma, USA), pH 7.5. After 4 hours, the buffer was changed and the dialysis continued overnight. The precipitate formed was separated by high speed centrifugation (10,000 × g/20 min/0° C), washed once with the EGTA buffer, and dissolved in 50 ml of a buffer consisting of 0.1 M Tris-HCl 0.2 M NaCl 0.01 M EDTA, pH 7.5. This solution was stored at −80° C. The EDTA was neutralized before use by addition of a 0.02 M $CaCl_2$ solution. b) Purification of the EGTA precipitate was performed on Sephadex G-200. The precipitate resulting from the dialysis of 50 ml swine serum in EGTA buffer was dissolved in 5 ml of the Tris buffer and transferred to a column containing Sephadex G-200 equilibrated with the same buffer to which 0 02 per cent NaN_3 was added. Column: K 26/40 (Pharmacia Fine Chemicals); bed diameter 2.6 cm; height: 40 cm; operating pressure: 10 cm H_2O. The effluent was recorded at 280 nm and fractions of 6 ml were collected. The fractions were examined for C1q activity by quantitative haemolytic assay and for enhancing effect on complement fixation of porcine IgG Furthermore, the fractions with the highest haemolytic C1q activity and effect on complement fixation were examined by immunoelectrophoresis c) Further purification of G-200 fractions was made on QAE-Sephadex. The two G-200 fractions with the highest C1q activity (12 ml) were concentrated to 2 ml by absorption in polyethylene glycol (M 20,000), dialysed in a buffer consisting of 0.1 M Tris-HCl 0.1 M NaCl 0.01 M EDTA, pH 7.5, and transferred to a column of QAE-Sephadex (A-50) equilibrated with the same buffer after addition of 0.02 per cent NaN_3. Column: K 9/15 (Pharmacia Fine Chemicals); bed volume: 10 ml. The effluent, which was eluted by means of a peristaltic pump, was recorded at 280 nm. 2 ml fractions were collected. After two protein peaks had been eluted, the column was washed with 20 ml starting buffer. A linear salt gradient was then poured into the column (0 1–1 M NaCl/300 ml). The collected fractions were examined in the same way as the G-200 fractions.

Haemolytic assay for C1q activity. Diluent, complement and haemolytic system were as described by *Schjerning-Thiesen* (17). Guinea pig complement depleted of C1q by dialysis in 0.018 M EGTA buffer was used as R-C1q. The EGTA was neutralized before use by adding a 0.02 M $CaCl_2$ solution. This reagent was completely without haemolytic activity in the amounts generally used. After addition of a solution of EGTA precipitate from guinea pig or swine serum, the complement activity was reconstituted.

The C1q activity in the different preparations was assayed by adding 0 1 ml of twofold dilutions to a constant amount of R-C1q (dilution 1/100 1.00 ml corresponding to two 50 per cent haemolytic units of whole complement) and veronal buffer to 2.40 ml. The mixtures were incubated at 37° C for 5 minutes and 1 60 ml haemolytic system was added. The incubation was continued for 60 minutes, upon which non-lysed erythrocytes were precipitated by centrifugation. O.D. was read at 523 nm. It was of crucial importance for the performance of this test that the sensitized erythrocytes were washed twice in veronal buffer before use, as described by *Lepow et al.* (12).

Haemolytic assay for C1 activity. The C1 activity in the C1q preparations was tested as described by *Lepow et al.* (12) with some modifications 0.1 ml of twofold dilutions was incubated with 1 60 ml haemolytic system and veronal buffer to 4.00 ml at 37° C for 30 minutes. After incubation, the mixtures were centrifuged, washed with 4 ml veronal buffer at 37° C and resuspended in 4.00 ml veronal buffer containing guinea pig complement (1/40) and Na_2Mg-EDTA (7.5 × 10^{-3} M). After further incubation at 37° C for 60 minutes, non-lysed erythrocytes were precipitated by centrifugation and O D. was read at 523 nm.

With a view to comparison, the C1q activity and the C1 activity were assayed in euglobulin fractions prepared from swine serum and guinea pig serum by dialysis in 0 02 M phosphate buffer, pH 5 4 (13).

Preparation of IgG from swine serum containing antibodies to pseudorabies virus. Three sera were used, two obtained from pigs inoculated intranasally with cell cultured pseudorabies virus and one obtained from a contact infected pig. 22.5 g $(NH_4)_2SO_4$ was added to 90 ml serum and the

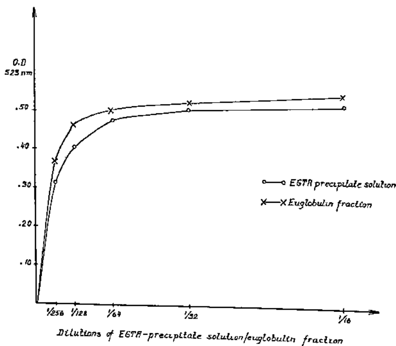


Fig. 1. Haemolytic assay for C1q activity of an EGTA precipitate solution and a euglobulin fraction from swine serum. Twofold dilutions were tested with a constant amount of R-C1q (dilution 1/100; 1.00 ml).

mixture was left at room temperature overnight under stirring. After centrifugation (8000 rev/min/30 min/20° C. JA-20 Beckman rotor), the supernatant was discarded and the sediment washed with 22.5 ml 1.75 M $(NH_4)_2SO_4$. The mixture was centrifuged (8000 rev/min/15 min). The antibody containing precipitate was dissolved in redistilled water to make 22.5 ml and dialysed overnight against a 0.01 M phosphate buffer pH 7.6. Interfering lipoproteins were precipitated by adding a 10 per cent dextran sulphate (1/50) and incubating at 0° C for 10 minutes under stirring. After addition of a 10 per cent $CaCl_2$ solution (1/10), incubation was continued for a further 10 minutes. The mixture was centrifuged (20,000 rev/min/30 min/5° C. JA-20 Beckman rotor). The supernatant was transferred to a column (K 16/40 Pharmacia Fine Chemicals) containing DEAE-cellulose (Whatman DE-52) equilibrated with a 0.01 M phosphate buffer, pH 7.6. Fractions from the excluded peak containing the IgG were dialysed overnight at 4° C against 0.1 M NaCl containing 0.02 per cent NaN_3.

Complement fixation technique. a) A direct complement fixation test using microtiter equipment was made (Cooke Engineering). The antigen was pseudorabies virus cultivated in primary swine kidney cell cultures. Medium from non-inoculated cell cultures was used as control antigen. The different IgG preparations served as immune serum. Four 100 per cent haemolytic units of complement were used with and without the addition of C1q preparation. The components, all in amounts of 0.025 ml, were mixed upon which the microplates were incubated at room temperature for 1 hour and subsequently overnight at 4° C. Thereafter, 0.025 ml of the haemolytic system was added. After incubation at 37° C for 30 minutes, the plates were left overnight at 4° C, upon which a final reading was made. The enhancing effect of the C1q preparations on the ability of porcine IgG to fix guinea pig complement was assayed by adding twofold dilutions of the C1q preparation to identical mixtures of IgG (2 units), antigen (2 units) and complement (4 units). Complement fixation did not take place in these mixtures except in the presence of a preparation of porcine C1q. b) A quantitative complement fixation method described by *Schjerning-Thiesen* (17) was used, with the modification that the mixtures of immune serum, antigen and complement were initially incubated for 60 minutes at 37° C before overnight incubation at 0° C. After incubation, the number of fixed complement units was determined quantitatively. Fifty 50 per cent haemolytic units of complement were used with and without the addition of C1q preparation.

Immunoelectrophoresis. Fractions containing C1q

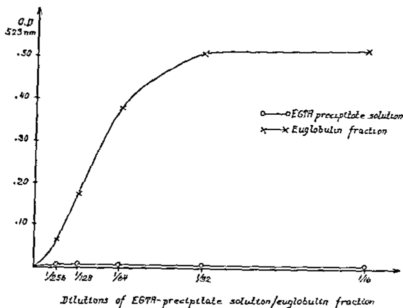


Fig. 2. Haemolytic assay for C1 activity of an EGTA precipitate solution and a euglobulin fraction from swine serum. Twofold dilutions were tested with veronal buffer containing guinea pig complement (1/40) and Na_2Mg-EDTA (7.5×10^{-3} M).

activity from the Sephadex G-200 and QAE-Sephadex separations were examined by crossed immunoelectrophoresis and rocket immunoelectrophoresis, as described by *Weeke* (21). The electrophoresis was performed with 1 per cent agarose gel in barbital buffer, pH 8.6, ionic strength 0.02. The antibody used was a rabbit antiswine serum protein (Dakopatts A/S, Copenhagen) and an immune serum produced in rabbits with fractions containing C1q activity from the QAE-Sephadex separation, using an immunization procedure described by *Harboe* & *Ingild* (10).

RESULTS

Haemolytic assay for C1q and C1 activity. The results of the quantitative haemolytic assay for C1q activity in an EGTA precipitate solution and euglobulin fraction from swine serum are shown in Fig. 1. Both preparations were able to reconstitute the haemolytic activity of R-C1q. The C1q activity was high and almost similar in the two preparations. In contrast, no C1 activity was found in the EGTA precipitate solution, while the euglobulin fraction showed a high C1 activity (Fig. 2). This points to the fact that the EGTA precipitate contains a high concentration of C1q and no demonstrable C1r or C1s, whereas the euglobulin fraction, according to expectation, contains the whole C1 complex.

Complement fixation test. If IgG preparations containing antibodies to pseudorabies virus were tested in a direct complement fixation test, no specific complement fixation would be seen unless a solution of the EGTA precipitate had been added to the complement. The antibody titres obtained were identical whether 2–10 per cent (v/v) EGTA precipitate solution was added to the complement diluent. Addition of the same amounts of EGTA precipitate prepared from guinea pig serum had no stimulating effect at all on the complement fixation.

Purification on Sephadex G-200. In order to obtain further knowledge of the active component (or components) in the EGTA precipitate, separation was made on Sephadex G-200. The elution profile of the gel filtration of 5 ml EGTA precipitate solution prepared from 50 ml swine serum is shown in Fig. 3. The C1q activity determined by haemolytic assay was found to be highest in fractions 14 and 15. In crossed immunoelectrophoresis

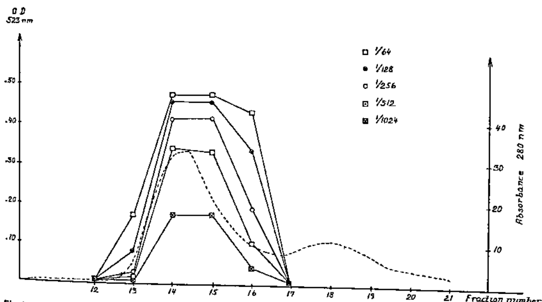


Fig. 3. Elution profile of Sephadex G-200 filtration of an EGTA precipitate solution. ------ = absorbance 280 nm. ——— = haemolytic C1q activity in different dilutions.

of a pool of these fractions, using rabbit anti-swine serum protein in the second dimension, at least two precipitates were seen (Fig. 4).

Further purification on QAE-Sephadex. The pool of fractions 14 and 15 (12 ml) was concentrated to 2 ml and transferred to a column containing QAE-Sephadex. The elution profile is shown in Fig. 5. Haemolytic

Fig. 4. Crossed immunoelectrophoresis of pool 14-15 from the Sephadex G-200 filtration, using rabbit antiswine serum protein in the second electrophoretic step. First dimension: Anode to the left. Second dimension: Anode at the top.

C1q activity was found only in the fractions excluded in the first peak. Correspondingly, a strong, stimulating effect on complement fixation by porcine IgG was found in the same fractions (Fig. 6). In crossed immunoelectrophoresis of these fractions, no precipitate was seen. Apparently, the protein did not enter the agarose gel under the conditions used. Using the rabbit antiswine serum protein in rocket immunoelectrophoresis, no precipitate was found; if, however, antiserum from rabbits immunized with the C1q containing fractions from the QAE-Sephadex separation was used, single rocket-shaped precipitates would be formed (Fig. 7). If the same antiserum was used for examination of whole swine serum, a similar single rocket-shaped precipitate would be formed (Fig. 7). These findings indicate that porcine C1q was obtained in a reasonable state of purity by this procedure.

Effect of QAE-Sephadex purified porcine C1q on complement fixation by porcine IgG-antibodies demonstrated by a quantitative technique. Varying amounts of IgG (dilution 1/10: 0.30, 0.60, 1.20 and 2.40 ml) were incubated with a constant amount of pseudorabies antigen/control antigen (0.25 ml un-

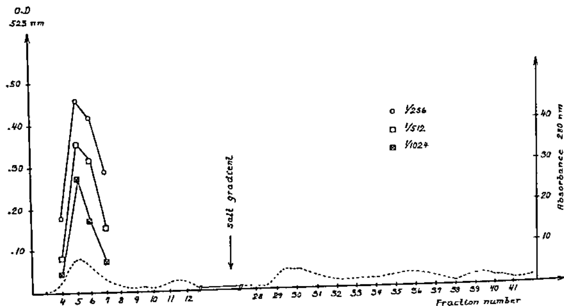
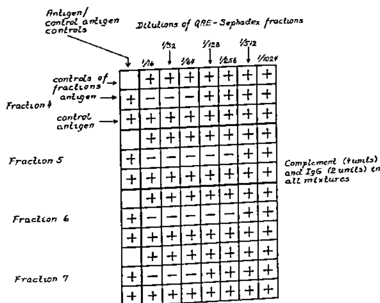


Fig. 5. Elution profile of QAE-Sephadex separation of pool 14/15 from the Sephadex G-200 filtration. - - - - - = absorbance 280 nm. ——— = haemolytic C1q activity in different dilutions.



	Antigen/control antigen controls	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
Fraction 4 – controls of fractions		+	+	+	+	+	+	+
Fraction 4 – antigen	+	−	−	−	+	+	+	+
Fraction 4 – control antigen	+	+	+	+	+	+	+	+
Fraction 5 – controls of fractions		+	+	+	+	+	+	+
Fraction 5 – antigen	+	−	−	−	−	−	+	+
Fraction 5 – control antigen	+	+	+	+	+	+	+	+
Fraction 6 – controls of fractions		+	+	+	+	+	+	+
Fraction 6 – antigen	+	−	−	−	−	−	+	+
Fraction 6 – control antigen	+	+	+	+	+	+	+	+
Fraction 7 – controls of fractions		+	+	+	+	+	+	+
Fraction 7 – antigen	+	−	−	−	+	+	+	+
Fraction 7 – control antigen	+	+	+	+	+	+	+	+

Fig. 6. Enhancing effect of twofold dilutions of QAE-Sephadex fractions on the complement fixation in identical mixtures of IgG-antibodies, antigen and complement. No fixation took place unless a preparation of porcine C1q was present. + = 100 per cent haemolysis; (+) = 50–100 per cent haemolysis; (—) = 0–50 per cent haemolysis, — = 0 per cent haemolysis

diluted), complement (0.25 ml undiluted = fifty 50 per cent haemolytic units) and C1q preparation (dilution 1/8:0.50 ml); total volume: 5.00 ml. It was found in preliminary experiments that the amount of C1q preparation used gave maximum fixation with the IgG. In a parallel experiment, identical mixtures were incubated without the addition of

Clq preparation. After overnight incubation, the number of complement units fixed in the mixtures was determined according to *Schjerning-Thiesen* (17).

In the absence of Clq preparation in the mixtures, no specific fixation was obtained by the two smallest amounts of IgG (0.30 and 0.60 ml), whereas five and seven units were fixed by 1.20 and 2.40 ml IgG dilution, respectively (Fig. 8). In the presence of Clq preparation, very strong overall fixation was obtained. Using the two largest amounts of IgG, the number of complement units fixed was at least five times the number to be fixed if porcine Clq was not present. Using the control antigen, there was no fixation in any of the experiments.

Furthermore, experiments were performed in which constant amounts of IgG (dilution 1/10: 1.00 ml), antigen/control antigen (0.25 ml) and complement (0.25 ml = fifty 50 per cent haemolytic units) were incubated with increasing amounts of Clq preparation. As will be seen from Fig. 9, the number of complement units to be fixed increased with increasing amounts of Clq preparation until a maximum was reached.

In the amounts used, the Clq was com-

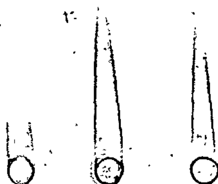


Fig. 7. Rocket immunoelectrophoresis of whole swine serum (left) and QAE-Sephadex fraction 5 (midst) and 6 (right), using antiserum from rabbits immunized with fractions containing Clq from the QAE-Sephadex separation. Anode at the top.

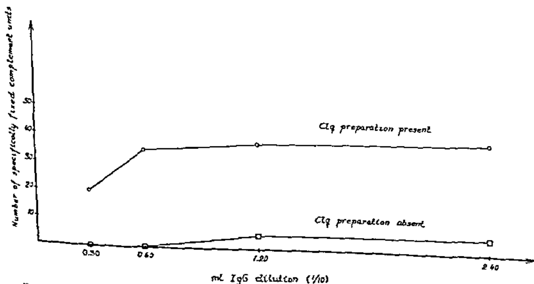


Fig 8. Results of quantitative complement fixation using fifty 50 per cent haemolytic units of complement, varying amounts of IgG-antibodies, and a constant amount of antigen in the presence and absence of Clq preparation.

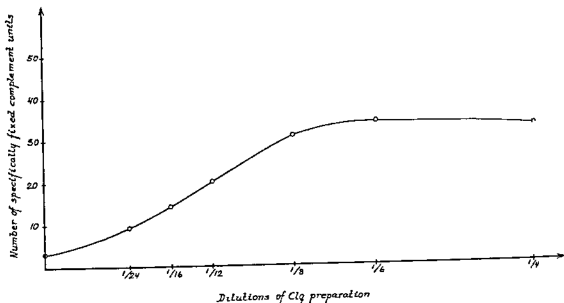


Fig. 9. Results of quantitative complement fixation using fifty 50 per cent haemolytic units of complement, varying amounts of QAE-Sephadex purified porcine C1q, and a constant amount of IgG-antibodies and antigen.

pletely inactivated by heat treatment at 56° C for 30 minutes, both as regards haemolytic activity and the effect on complement fixation.

DISCUSSION

If direct complement fixation tests using four units of complement were made in microplates, using porcine IgG as antibody source, no complement fixation was seen unless a preparation of porcine C1q was present. In the quantitative test, a few units of complement were fixed by the largest amounts of IgG in the absence of porcine C1q. However, even if the large amounts of IgG were used, the number of complement units to be fixed was at least five times higher in the presence of porcine C1q.

The clear effect of the C1q preparations on the complement fixation points to a selective requirement of porcine antibody (IgG)-antigen complexes for porcine C1q as recognition unit, as opposed to guinea pig C1q. The activation of the complement probably proceeds through guinea pig C1r and C1s in the same way as the porcine C1q is able to reconstitute the haemolytic activity of guinea pig complement depleted of C1q.

Incompatibility of avian antibody-antigen complexes with guinea pig complement has been described (3, 22). *Benson et al.* (3) considered that incompatibility existed between certain avian antibody-antigen complexes and the C1 component of guinea pig complement. *Olsen et al.* (15) demonstrated incompatibility between feline antibody-antigen complexes and guinea pig C1. In a recent report, *Volanakis & Kaplan* (19) described a requirement for human C1q in the consumption of guinea pig complement by C-reactive protein complexes. Sheep erythrocytes sensitized with nurse shark antibody (16) were incompatible with guinea pig C1, but in the presence of shark C1, a complex was formed that could be lysed with guinea pig C4–C9.

Since the solubility of human C1q is minimal at low ionic strength, highly purified C1q can be obtained from human serum by repeated precipitation in the presence of chelating agents by which to eliminate C1r and C1s (23). With porcine serum, the best yields of C1q were obtained by initial precipitation followed by separation on Sephadex G-200

and QAE-Sephadex. The elution patterns of the C1q activity in these separations reveal that, in this respect, the porcine C1q resembles human C1q (14, 1). On the basis of the immunoelectrophoretic examinations, it seems likely that porcine C1q can be obtained in a reasonable state of purity by QAE-Sephadex separation. It is suggested that the commercial rabbit antiswine serum protein does not contain antibodies to C1q sufficient to form a precipitate under the conditions used. Further studies on the characterization of the porcine C1q are necessary.

Investigations are in progress using the porcine C1q in the complement fixation test with whole swine immune sera. By addition of this component to the complement, improved reactions can be obtained, but there still seems to be a serum factor (or factors) which has some inhibiting effect on the complement fixation. A subsequent paper will describe the destruction of this factor by mercapto-ethanol treatment.

REFERENCES

1. *Assimeh, S. N., Bing, D. H. & Painter, R. H.:* A simple method for the isolation of the subcomponents of the first component of complement by affinity chromatography. J. Immunol. *113*: 225–234, 1974.
2. *Bankowski, R. A., Wichmann, R. & Kummer, M.:* A complement fixation test for identification and differentiation of immunological types of the virus of vesicular exanthema of swine. Amer. J. Vet. Res. *14*: 145–149, 1953.
3. *Benson, H. N., Brumfield, H. P. & Pomeroy, B. S.:* Requirement of avian C1 for fixation of guinea pig complement by avian antibody-antigen complexes. J. Immunol. *87*: 616–622, 1961.
4. *Boulanger, P.:* Complement-fixation tests of swine serum. I. In the diagnosis of vesicular stomatitis. Can. J. Comp. Med. *19*: 37–47, 1955.
5. *Boulanger, P., Appel, M., Bannister, G. L., Ruckerbauer, G. M., Mori, K. & Gray, D. P.:* Hog cholera. III. Investigation of the complement-fixation test for the detection of the virus in swine tissue. Can. J. Comp. Med. *29*: 201–208, 1965.
6. *Boulanger, P., Bannister, G. L., Gray, D. P., Ruckerbauer, G. M. & Willis, N. G.:* African swine fever. II. Detection of the virus in swine tissues by means of a modified direct complement-fixation test. Can. J. Comp. Med. *31*: 7–11, 1967.
7. *Cowan, K. M.:* Immunological studies on African swine fever virus. I. Elimination of the procomplementary activity of swine serum with formalin. J. Immunol. *86*: 465–470, 1961.
8. *Cowan, K. M.:* Immunologic studies on African swine fever virus. II. Enhancing effect of normal bovine serum on the complement-fixation reaction. Amer. J. Vet. Res. *24*: 756–761, 1963.
9. *Eskildsen, M.:* Studies on the procomplementary effect of swine serum. Arch. Exp. Vet. Med. *27*: 115–123, 1973.
10. *Harboe, N. & Ingild, A.:* In: Axelsen, N. H., Krøll, J. & Weeke, B. (Eds.): A manual of quantitative immunoelectrophoresis. Methods and applications. Scand. J. Immunol. *2*, suppl. 1: 161–164, 1973.
11. *Knight, G. J. & Cowan, K. M.:* Studies on allegedly non-complement-fixing immune systems. I. A heat labile serum factor requirement for a bovine antibody complement-fixing system. J. Immunol. *86*: 354–360, 1961.
12. *Lepow, I. H., Naff, G. B., Todd, E. W., Pensky, J. & Hinz, C. F.:* Chromatographic resolution of the first component of human complement into three activities. J. Exp. Med. *117*: 983–1008, 1963.
13. *Mayer, M. M.:* In: Kabat, E. A. & Mayer, M. M. (Eds.): Experimental Immunochemistry, Charles C Thomas, Springfield, 2nd Ed., 2nd Pr., 1964, p. 162.
14. *Müller-Eberhard, H. J.:* Chemistry and reaction mechanisms of complement. In: Dixon, Jr., F. J. & Kunkel, H. G. (Eds.): Advances in Immunology, Vol. 8, 1–80, Academic Press, New York, 1968.
15. *Olsen, R. G., Krakowka, S. G., Mathes, L. E. & Yohn, D. S.:* Composite complement: A mixture of feline C1 and guinea pig complement as a reagent compatible with feline antibodies. Amer. J. Vet. Res. *35*: 1389–1392, 1974.
16. *Ross, G. D. & Jensen, J. A.:* The first component (C1n) of the complement system of the nurse shark (*Ginglymostoma cirratum*). I. Hemolytic characteristics of partially purified C1n. J. Immunol. *110*: 175–182, 1973.
17. *Schjerning-Thiesen, K.:* Studier over komplement-binding. En forbedret kvantitativ komplementbindingsteknik ved diagnosticering af aphtae epizooticae. Thesis, A/S C. Fr. Mortensen, Copenhagen, 1964.
18. *Switzer, W. P.:* Mycoplasmal pneumonia of swine. J. A. V. M. A. *160*: 651–653, 1972.
19. *Volanakis, J. E. & Kaplan, M. H.:* Interaction of C-reactive protein complexes with

the complement system. II. Consumption of guinea pig complement by CRP complexes: Requirement for human C1q. J. Immunol. *113:* 9–17, 1974.

20. *Wallis, A. S. & Thompson, G. W.:* The evaluation of a complement fixation test for the diagnosis of porcine enzootic pneumonia. Vet. Rec. *85: 573–577, 1969.*

21. *Weeke, B.:* In: Axelsen, N. H., Kroll, J. & Weeke, B. (Eds.): A manual of quantitative immunoelectrophoresis. Methods and applications. Scand. J. Immunol. *2,* suppl. 1: 37–56, 1973.

22. *Wilcox, G. E. & Consigli, R. A.:* Complement-fixation test for detection of viral antibodies in chicken serum. I. Standardization of the test for detection of antibodies to Newcastle disease virus and polyoma virus. Avian Dis. *16: 1011–1018, 1972.*

23. *Yonemasu, K. & Stroud, R. M.:* C1q: Rapid purification method for preparation of monospecific antisera and for biochemical studies. J. Immunol. *106:* 304–313, 1971.

Acta path. microbiol. scand. Sect. C, *83:* 325–327, 1975

BRIEF REPORT

ANTIGENICITY OF HUMAN IgM IN RELATION TO INTERACTION WITH STAPHYLOCOCCAL PROTEIN A

A. Grov

The University of Bergen, School of Medicine, The Gade Institute, Department of Microbiology, Bergen, Norway

Grov, A. Antigenicity of human IgM in relation to interaction with staphylococcal protein A. Acta path. microbiol. scand. Sect. C, *83:* 325–327, 1975.

Antibodies to protein A reactive and non-reactive human IgM proteins were produced in rabbits. Serological examinations revealed antigenic determinant(s) of the protein A reactive IgM not present on the non-reactive IgM. Isolated antibodies to this determinant(s) were shown to be capable of inhibiting interaction with protein A. Three IgM preparations from different pools of normal human sera were all found to contain protein A reactive fractions. The observed difference in protein A reactivity points to an isotype or subclass deviation of human IgM.

Key words: Human IgM; antigenicity; staphylococcal protein A; interaction with.

A. Grov, Mikrobiologisk avdeling, MFH-bygget, N-5016 Haukeland sykehus, Norway.

Received 9.vi.75 Accepted 9.vi.75

In a previous paper (2) three of seven IgM proteins from sera of patients with macroglobulinemia were found to interact with staphylococcal protein A. They gave co-precipitation and the "star-phenomenon", but no direct precipitation in agar. The primary protein A reactive site(s) was located to the Fc-region, but the co-precipitation and "star"-formation were also dependent on other molecular configurations.

The present paper deals with an immunological study of protein A reactive and protein A non-reactive IgM proteins, in the expectation that the difference in property would be reflected in their antigenicity.

Materials and Methods

IgM preparations. Four IgM proteins from the previous study (2), two being protein A reactive (IgM_{As} and IgM_{1775}) and two protein A non-reactive (IgM_{9628} and IgM_{8415}), were included. IgM was also isolated from three different pools of normal human serum (2 ml from each of 20 sera). The serum pools were dialysed against distilled water in the cold, and the precipitates formed were fractionated on a column of Sephadex G-200 (2.3 × 90 cm) in PBS containing 1 M NaCl. The IgM fractions were checked for purity against specific antisera, and protein concentrations measured as described in (5).

Subunits (7S) of IgM_{As}, Fab_{μ} and $(Fc)_{5\mu}$ fragments and normal human and rabbit IgG were those prepared and described earlier (2).

Antisera. Antibodies to IgM_{As} and IgM_{9628} were raised in rabbits given the first injection of protein (5 mg in 0.5 ml of saline) mixed with an equal volume of complete Freund's adjuvant (Difco Lab., USA) in one hind foot pad. The same amount of protein in incomplete Freund's adjuvant (Difco) was given intramuscularly three and five weeks later, the animals being bled one week after the last injection. Antisera specific to μ, α and γ heavy chains were obtained from Hyland, Belgium.

Immunoadsorbents. Columns of protein A and isolated IgM coupled to Sepharose 4B (Pharmacia, Sweden) were prepared and used as before (1).

Fab'-fragments of rabbit IgG. IgG was precipitated in 1.33 N $(NH_4)_2SO_4$ and digested with pepsin (Sigma, USA) in 0.1 M acetate buffer, pH

TABLE 1. *Haemagglutination Inhibition*

Haemaggl. system		Inhibitor			
Antigen	Serum	IgM_{As}	IgM_{9628}	IgM_{8638}	IgM_{1779}
IgM_{As}	anti-IgM_{As}	+	—	—	+
IgM_{As}	anti-IgM_{9628}	+	+	+	+
IgM_{9628}	anti-IgM_{9628}	+	+	+	+
IgM_{9628}	anti-IgM_{As}	+	+	+	+

+: Inhibition, —: No inhibition.
Four agglutinating units of sera: inhibiting concentrations of about 5 μg per ml.

4.0, containing 5 mM NaCl, for 18 h at 37° C, the substrate: enzyme ratio being 100:3. After fractionation on a column of Sephadex G-150, equilibrated with 0.1 M Tris-HCl, pH 7.8, containing 0.2 M NaCl and 2 mM EDTA · Na_2, the $F(ab')_2$ fragments were reduced by 0.2 M 2-mercaptoethanol at pH 8.0 and room temperature in the dark for 18 h, alkylated by incubation with 0.3 M iodoacetamide for 3 h at 37° C, and finally dialysed against saline buffered to pH 8.0.

Serological tests. Indirect haemagglutination, using tanned sheep erythrocytes (TSE), and inhibition of this reaction were carried out as described in (3). The amount of antigen employed for sensitization was 0.1–0.2 mg per 20 ml of 0.5 per cent TSE. Double diffusion in agar and tests for co-precipitation and the "star"-formation were carried out as previously (2). In inhibition studies of the two latter tests the IgM protein was incubated with the inhibitor 1–3 h at room temperature prior to the set-up.

A sensitive method to detect protein A reactivity of immunoglobulins was demonstrated by adding TSE sensitized with the protein to be tested to a twofold serial dilution of protein A.

Results

The two IgM proteins used for immunization (IgM_{As} and IgM_{9628}) showed a reaction of partial identity on double diffusion in agar, the line between IgM_{As} and homologous serum spurring weakly over that between IgM_{9628} and the corresponding antiserum.

In indirect haemagglutination cross-reaction was observed between all IgM proteins, both antisera being of high titres. All systems showed titres of 1/5120 except the IgM_{As}-anti-IgM_{As} system, which had a titre of 1/10240. TSE sensitized with a protein A reactive IgM were agglutinated by protein A in concentrations of 0.5 μg per ml or higher, whereas no agglutination was obtained with TSE sensitized with protein A non-reactive IgM proteins.

Haemagglutination inhibition studies, like double diffusion in agar, revealed a difference between the antisera to protein A reactive and to protein A non-reactive IgM (Table 1). Whereas the former proteins inhibited both homologous and heterologous haemagglutination systems, the latter did not inhibit TSE sensitized with a protein A reactive IgM in anti-IgM_{As} serum. This indicates the presence of antigenic determinant(s) on protein A reactive IgM in addition to those present on protein A non-reactive IgM. Reduced and alkylated IgM_{As} (7S subunits) also inhibited agglutination of IgM_{As}-sensitized TSE in anti-IgM_{As} serum, whereas both $(Fc)_{5\mu}$ and Fabμ, alone or together with IgM_{9628}, were inactive in this context.

Antibodies to the additional determinant(s) on IgM_{As} compared to IgM_{9628} were isolated on an immunoadsorbent column in which IgM_{9628} was covalently linked to Sepharose. By running IgG from rabbit anti-IgM_{As} through the column, common antibodies were fixed to the column leaving the specific IgM_{As} antibodies in the eluate. The possibility that these latter antibodies (or some of them) were directed against the protein A reactive site(s) of the IgM protein was then studied. To avoid protein A-Fc interactions and agglutination of TSE sensitized with IgM_{As} with the specific IgM_{As} antibodies, Fab' fragments were prepared. The Fab' fragments from specific IgM_{As} antibodies were found to inhibit the co-precipitation as well as the "star"-formation. The agglutination by protein A of TSE sensitized with IgM_{As} was also completely inhibited by these fragments. Fab' fragments of antibodies bound to the immunoadsorbent were inactive in these inhibition experiments. This indicates that at least some of the antibodies specific to IgM_{As} are directed against the protein A reactive site(s) of the molecule.

All the three IgM proteins isolated from the pools of normal human sera contained protein A reactive fractions, approximately 35, 66, and 67 per cent, respectively. The interaction of these IgM preparations with protein A was also inhibited by the Fab'-fragments of specific anti-IgM_{As} antibodies.

Discussion

The inhibition of indirect haemagglutination clearly demonstrated the presence of an extra antigenic determinant(s) on protein A reacting IgM, compared to non-reacting IgM proteins. The corresponding antibodies (or some of them) were shown to be directed against the site(s) of protein A interaction. Since these antibodies could not be blocked by a non-reactive IgM together with $(Fc)_{5\mu}$ of a reactive IgM, a conformational determinant(s) is most probably involved. The possibility that both Fab and Fc regions are involved in this context cannot be excluded.

The presence of an extra antigenic determinant(s) on protein A reacting IgM, compared to non-reacting IgM proteins, would indicate allotype variations. However, the fact that some sera contained both types of IgM (2) and that both types were demonstrated in pooled normal human sera, most probably points to an isotype or subclass deviation of human IgM. This was also the conclusion of *Harboe* & *Fölling* (4) although their basis for differentiation apparently was a property other than interaction with protein A.

References: 1. *Grov, A.:* Acta path. microbiol. scand. Sect. A, Suppl. *236:* 77–83, 1973.—2. *Grov, A.:* Acta path. microbiol. scand. Sect. C, *83:* 173–176, 1975.—3. *Grov, A.* & *Oeding, P.:* Acta path. microbiol. scand. Sect. B, *79:* 539–544, 1971.—4. *Harboe, M.* & *Fölling, I.:* Scand. J. Immunol. *3:* 471–482, 1974.—5. *Lowry, O. H., Rosebrough, N. J., Farr, A. L.* & *Randall, R. L.:* J. biol. Chem. *193:* 265–275, 1951.

Acta path. microbiol. scand. Sect. C, *83:* 329–337, 1975

FURTHER STUDIES OF THE CROSS-REACTION BETWEEN *KLEBSIELLA* TYPE 12 AND TYPE 13 CAPSULAR POLYSACCHARIDES IN SERA FROM HYPERIMMUNIZED RABBITS

Jorunn Eriksen

Kaptein W. Wilhelmsen og Frues Bakteriologiske Institutt, Rikshospitalet, Oslo 1, Norway

Eriksen, J. Further studies of the cross-reaction between *Klebsiella* type 12 and type 13 capsular polysaccharides in sera from hyperimmunized rabbits. Acta path. microbiol. scand. Sect. C, *83:* 329–337, 1975.

A cross-reaction between *Klebsiella* types 12 and 13 polysaccharides was demonstrated in hyperimmunized rabbit sera. In most of the sera, the cross-reaction could only be demonstrated by passive haemagglutination and not by precipitation. Immunization with *Klebsiella* type 13 induced antibodies of both the IgM and IgG class, but the antibodies cross-reacting with *Klebsiella* type 12 polysaccharide were only IgM antibodies. In *Klebsiella* type 12 sera, the antibody activity was mainly, or only, demonstrated in the IgM class, none or only traces being found in IgG. *Klebsiella* type 12 antigen seemed to inhibit or delay production of IgG antibodies.

Key words. *Klebsiella* type 12, type 13; capsular polysaccharides; cross-reactions; rabbit immune sera.

J. Eriksen, Kaptein W. Wilhelmsen og Frues Bakteriolgiske Institutt, Rikshospitalet, Oslo 1, Norway.

Received 11.ii.75 Accepted 2.iv 75

By examination of human monoclonal IgM proteins, 10 out of 116 sera showed antibody activity against the type specific capsular polysaccharides isolated from 17 different *Klebsiella* types (6). Three sera, Ro, We and Th were further investigated (5). The most interesting observation was the fact that the monoclonal IgM protein in serum Ro reacted with the capsular polysaccharide isolated from *Klebsiella* type 12 *and* type 13. It was demonstrated that the same IgM-molecules precipitated both polysaccharides to the same extent which indicated that the two type specific polysaccharides must have a cross-reacting common antigenic determinant.

Earlier examinations of the *Klebsiella* group did not show any clear cross-reaction between the two types, neither by tube agglutination nor by capsular swelling (1), (8), (10). By the passive haemagglutination test, however, a cross-reaction was demonstrated (7) in rabbit antisera. Specific antisera against *Klebsiella* type 12 agglutinated

sheep red blood cells (SRBC) coated with polysaccharide type 13 (PS13) to some extent. In antisera against *Klebsiella* type 13 SRBC coated with polysaccharide from type 12 (PS12) reacted more strongly. This was in contrast to the monoclonal IgM in serum Ro which reacted with both polysaccharides to the same titre.

The observations in the case of serum Ro and the rabbit immune sera indicated that a study of the specificity of antibodies produced in the course of prolonged immunization of rabbits with Klebsiella type 12 and type 13, might be of interest.

MATERIAL AND METHODS

Bacteria. Klebsiella types 12 and 13 were the same strains as those used in the previous studies (5), (7). The bacteria were grown in broth for 18 h and the culture was used for immunization as described earlier (7).

Immunization. Six random-bred rabbits were used for immunization. Before the immunization started, blood samples were collected to serve as zero value. The rabbits were injected intravenously, rabbits no. 31, 32 and 33 with type 12, and rabbits 34, 35 and 36 with type 13. Injections were given every 3 days for 5 weeks, and then once a week for a total of approx. 40 weeks. Small blood samples were collected from the ear vein every second week, larger amounts by heart puncture once a month. All sera were inactivated at 56° C for ½ h before use. The samples were numbered in succession as collected.

The capsular, type specific polysaccharide from the two *Klebsiella* types was isolated as described earlier (2). The polysaccharides (PS12 and PS13) were used as antigen in gel diffusion tests and for coating of SRBC in the haemagglutination tests. They were dissolved in saline (1 mg/ml).

The passive haemagglutination procedure was performed as before (3)

Separation of antibodies by ultracentrifugation was carried out using a 10 to 40 per cent (w/v) sucrose gradient in saline. 0.3 ml serum diluted 1:2 was layered on top of the gradient (4.4 ml) and the centrifugation proceeded for 18 h at 5° C at 36,000 rev/min in a swinging bucket rotor in an MSE 65 ultracentrifuge After the end of the run, fractions were collected from the bottom of the centrifuge tube by punching a hole in the tube, giving the 19 S components close to the bottom and the 7 S in the upper half of the tube. About 18 fractions, each of 4 drops, were collected from each tube. Each fraction was examined by the passive haemagglutination procedure.

Gel diffusion test was made in 1 per cent agar in saline.

Quantitative precipitation was carried out in one serum with the homologous polysaccharide as antigen, using the Biuret method for determination of protein (9).

Electrophoresis on cellulose acetate membranes was performed as described in an earlier paper (4).

EXPERIMENTS AND RESULTS

Sera from rabbits under hyperimmunization were examined continuously during an immunization period of approx. 40 weeks Blood samples drawn at different intervals were examined by gel diffusion and by haemagglutination. The results are reported in Table 1 and 2.

Table 1 gives the results obtained by examination of serum samples from rabbit no. 31 hyperimmunized with *Klebsiella* type 12. Examination of the serum samples from rabbits nos. 32 and 33 in the same group, showed the same pattern.

Most sera from rabbits nos. 31, 32 and 33, immunized with type 12, showed positive gel precipitation against PS12 But none of the sera gave a positive gel precipitation with PS13, not even sera from the latter part of the immunization period with strong homologous reaction.

By passive haemagglutination, on the other hand, positive reactions were obtained with both polysaccharide antigens, even though the reactions with the heterologous polysaccharide were rather weak.

Rabbits nos. 34, 35 and 36, immunized with *Klebsiella* type 13, gave different results. Table 2 reports the results from examination of rabbit no. 36. The table also represents the pattern obtained by examination of rabbits nos. 34 and 35. The gel precipitation against PS13 was very strong. In some of the serum samples the antibodies also showed positive gel precipitation with PS12 (Fig. 1). The precipitation line between serum and PS12, however, seemed to be inhibited to reach the line between serum and PS13.

The haemagglutination titres in this group were much higher compared with those in

TABLE 1. *Antibody Response to* Klebsiella *Type 12 and Type 13 of Rabbit No. 31 Hyperimmunized with* Klebsiella *Type 12*

Serum sample no.	No. of injections and days after start of immuniz.		Haemagglutination titre* against		Gel precipitation against PS12	Electrophoresis of antiserum on cellulose acetate membranes
			type 12	type 13		
301	0	0	4	4	—	
307§	3	12	32	32	(+)	
313	5	19	128	128	(+)	
319	6	24	128	128	+	
325§	8	32	256	128	+	Antibodies with restricted electrophoretic heterogeneity can be seen in serum 325 and 331. One band.
331	10	43	256	64	+	
337§	12	52	1024	32	+	
343	15	64	512	32	+	
349§	21	100	512	32	++	
354§	27	142	6144	128	++	Antibodies with restricted heterogeneity are also present in serum 354, 359 and 364. One strong band. In serum 369 and 374 the narrow band is still present, but not so strong.
359§	30	169	8192	64	++	
364	34	198	8192	64	++	
369	39	242	2048	32	++	
374§	42	270	6144	128	++	

* Reciprocal of highest dilution giving distinct haemagglutination.
§ These sera have been examined by sucrose gradient ultracentrifugation.

TABLE 2. *Antibody Response in* Klebsiella *Type 12 and Type 13 of Rabbit No. 36 Hyperimmunized with* Klebsiella *Type 13*

Serum sample no.	No. of injections and days after start of immuniz.		Haemagglutination titre* against		Gel precipitation against PS13	Electrophoresis of antiserum on cellulose acetate membranes
			type 12	type 13		
306	0	0	8	4	—	
312§	3	12	1024	1024	(+)	
318	5	19	2048	2048	+	
324§	6	24	2048	2048	+	
330	8	32	2048	4096	+	
336§	10	43	1024	2048	+	
342	12	52	256	2048	+	
348§	15	64	384	3072	+	
353§	21	100	384	1024	+	In the last three serum samples, antibodies with restricted electrophoretic mobility could be demonstrated as one strong line.
358§	26	142	768	16384	++$	
363	29	169	1024	16384	++$	
368§	33	198	4096	32768	++$	

* Reciprocal of highest serum dilution giving distinct haemagglutination.
§ These sera have been examined by sucrose gradient ultracentrifugation.
$ The serum sample showed also positive gel precipitation against PS12.

the other group. The haemagglutination titres against the heterologous polysaccharide sometimes were just as high, or even higher than those against the homologous type, especially early in the immunization period.

All rabbits, either immunized with *Klebsiella* type 12 or type 13 sooner or later produced antibodies of restricted electrophoretic mobility (Tables 1 and 2, last column).

Some serum samples from the six rabbits were fractionated by sucrose gradient ultracentrifugation into 18 fractions, each of

21*

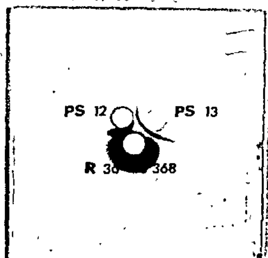


Fig. 1. Double diffusion in agar of anti-*Klebsiella type 13 serum against PS12 and PS13.*

which was examined by passive haemagglutination for antibodies against *Klebsiella* type 12 *and* type 13 polysaccharides. The results are recorded in Table 3.

The tables show only the haemagglutination titres from one rabbit in each group. Serum samples from the other rabbits in the two groups showed the same distribution of antibody activity. By comparing the results from the two groups, there is a striking difference in the distribution of the activity. In serum samples from rabbits immunized with *Klebsiella* type 12, the activity seemed to be located in the first ten fractions, with the main activity in fractions nos. 3, 4 and 5, in the IgM class. During an immunization period of approx. 40 weeks, the haemagglutination titre against PS12 increased more rapidly than the titre against PS13, but all activity remained in the IgM antibodies.

As regards the three rabbits immunized with *Klebsiella* type 13, the antibody activity against PS12 and PS13 seemed different. The activity against the heterologous PS12 started and remained in the IgM class, while the activity against the homologous PS13 started in the IgM class to switch over to the IgG class later in the immunization period.

Figs. 2 and 3 illustrate the results obtained with 3 sera from 1 rabbit in each group. The marked difference between the two types of antibody production is plainly demonstrated. The sera illustrated in Fig. 2 contained antibodies with restricted heterogeneity. This is demonstrated in Fig. 4. Serum 301 was drawn before the immunization started. Comparison of Fig. 2 and Fig. 4 indicates that the antibodies with restricted heterogeneity must be of the IgM class.

Antibodies with restricted heterogeneity

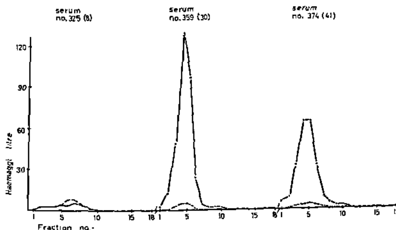


Fig. 2. Distribution of haemaggl. titres in the fractions after sucrose gradient ultracentrifugation of serum dilutions from rabbit 31, anti-Klebsiella type 12. Figures in parentheses indicate number of immunization. Full line PS12. Broken line: PS13.

TABLE 3 *Distribution of Haemagglutination Activity against PS12 and PS13 after Ultracentrifugation of Serum Dilutions from Rabbit no. 31 Immunized with Klebsiella Type 12, and from Rabbit no. 36 Immunized with Klebsiella Type 13*

Serum no	Antigen	Haemagglutination titre* in fraction no. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Rabbit no. 31																			
307	PS12			1	2	4	2	2											
	PS13				4	4	4	2											
325	PS12		1	2	2	4	4	6	4	2									
	PS13	1	2	2	2	4	8	8	4	2	1								
337	PS12	1	2	4	16	16	12	2	1										
	PS13	1	1	2	3	4	8	2	1										
349	PS12			6	24	16	6												
	PS13			2	4	4	2												
354	PS12			2	4	4	2												
	PS13			2	8	16	8	2	2										
359	PS12	1	12	48	128	96	24	4	1	1									
	PS13			2	4	3	1												
374	PS12	6	8	32	64	64	32	8	4	2	2								
	PS13			1	2	3	2												
Rabbit no. 36:																			
312	PS12		2	8	32	32	16	4	1										
	PS13	2	6	16	32	64	32	4	3	8	12	16	16	8	4				2
324	PS12		1	4	8	16	16	4	2										
	PS13	1	3	8	24	64	64	8	6	6	8	32	32	12	4	2	4	4	
336	PS12		2	16	32	32	8	3	2	1	1								
	PS13	2	6	32	64	32	16	4	6	64	128	64	16	8	4	4	4	8	
348	PS12		2	8	16	16	16	8	2										
	PS13	2	3	6	12	32	16	16	8	16	32	64	24	24	24	4	4	2	
353	PS12		1	8	24	16	4	1											
	PS13	1	3	16	32	32	8	8	64	64	96	128	64	16	16	16	16		
358	PS12		8	32	24	3	1	2	3	2	2								
	PS13		1	8	12	32	12	4	4	32	164	128	512	128	128	32	8	8	2
368	PS12		8	16	8	4	2	2	2	2	2								
	PS13		1	2	2	1	1	1	12	48	192	384	1536	768	48	6	6	6	4

* Reciprocal of highest serum dilution giving distinct haemagglutination.

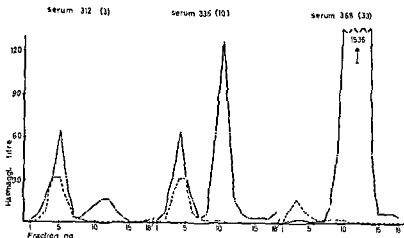


Fig. 3. Distribution of haemaggl. titre in the fractions after sucrose gradient ultracentrifugation of serum dilutions from rabbit 36, anti-*Klebsiella* type 13. Figures in parentheses indicate number of immunization. *Full line: PS13. Broken line: PS12.*

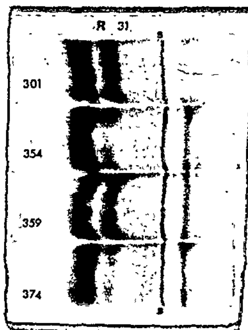


Fig. 4. Electrophoresis on cellulose acetate of anti-*Klebsiella* type 12 sera from rabbit 31. Serum 301 *is taken before immunization started. s: indicates* the starting line

were also demonstrated in Fig. 5, in serum samples from rabbit no. 36, anti-*Klebsiella* type 13. The antibodies with restricted heterogeneity in serum 368 belong to the IgG class. By comparing the electrophoretic mobility, the homogenous antibodies in serum 368 move further towards the catode than the IgM *antibodies in serum from rabbit no. 31.*

To confirm that the antibodies with restricted heterogeneity were specific antibodies against the capsular polysaccharides, two sera mentioned above were examined in the following way: To 1 ml of serum 368, 500 μg PS13 *was added, and to another serum sample* 500 μg PS12 *was added.* In both

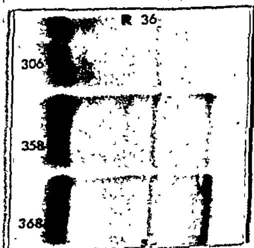


Fig. 5. Electrophoresis on cellulose acetate of anti-*Klebsiella type* 13 sera from rabbit 36. Serum 306 is taken before immunization.

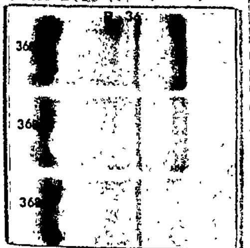


Fig. 6. Electrophoresis on cellulose acetate of anti-*Klebsiella* type 13 serum. 368 a: Serum before precipitation. 368 b: Supernatant after precipitation with PS12. 368 c. Supernatant after precipitation with PS13.

cases precipitation occurred. The mixtures were left in the cold for five days before centrifugation. Electrophoresis on cellulose acetate of serum was compared with electrophoresis of the two supernatants after the precipitations.

The results in Fig. 6 demonstrate that, when the homologous polysaccharide is added, the bands representing antibodies with restricted heterogeneity disappear from serum. On the other hand, antibodies with restricted heterogeneity remained in the supernatant after precipitation with PS12.

The same experiment was carried out in serum 374, but the electrophoresis showed another pattern (Fig. 7). Electrophoresis showed immunoglobulins with restricted heterogeneity, but this fraction was not removed from serum either by the homologous PS12 or by PS13.

Since these experiments were carried out without taking into consideration the amount of polysaccharide added to the serum, the supernatant might therefore contain a large excess of polysaccharide which inhibited the precipitation. A quantitative precipitation was carried out in serum 374. The supernatants were examined with regard to passive haemagglutination with PS12 and PS13. The results are reported in Table 4.

According to this table, 80 μg PS12 seemed to precipitate the maximum amount of antibodies in 1 ml serum. This amount was chosen for the precipitation, and the supernatant was examined for restricted heterogeneity once more. But the electrophoresis gave the same result. The supernatant still contained antibodies with restricted heterogeneity. Some haemagglutination activity is also left in the supernatant.

DISCUSSION

By means of the passive haemagglutination test, a cross-reaction between *Klebsiella* type 12 and type 13 was confirmed. A striking difference, however, between the immune responses to the two *Klebsiella* types was demonstrated. Throughout the immunization period of up to 9 months, antibodies, homologous as well as cross-reacting, in sera against *Klebsiella* type 12 remained in the IgM



Fig. 7. Electrophoresis on cellulose acetate of anti-*Klebsiella* type 12 serum. 374a: Serum before precipitation. 374 b: Supernatant after precipitation with PS12. 374 c: Supernatant after precipitation with PS13.

TABLE 4. *Haemagglutination Titre in Supernatant after Precipitation with Homologous Polysaccharide in Serum from Rabbit no. 31, Anti-*Klebsiella *Type 12, Using PS12 and PS13 as Antigens*

Microgram PS12 added to 1 ml of serum	Microgram antibody N precipitated in 1 ml serum	Haemagglutination titre in supernatant with	
		PS12	PS13
5	22	256	32
10	36	192	16
25	67	48	8
50	80	16	16
80	83	8	16
100	80	12	16

class. Only two serum samples from one rabbit showed a very slight activity against the homologous antigen in fractions likely to represent IgG. It should be borne in mind that some slight activity might have been missed, due to the dilution of the sera in connection with the ultracentrifugation.

The failure of precipitation with the homologous antigen to remove the bands of immunoglobulins with restricted mobility from anti-type 12 serum, and to remove all haemagglutinating activity, are observations which must remain unexplained at the present time.

The antibody production against *Klebsiella* type 13 showed a different pattern. The antibody production started with the IgM class, but later the homologous antibody production was switched over to the IgG class whereas the antibodies cross-reacting with PS12 remained in the IgM class. It is another observation that these antibodies apparently failed to react with the homologous antigen.

The six rabbits were random-bred and were identically immunized with identical quantities of broth cultures. The only difference between the two groups is the nature of the antigen. The number of rabbits was small, but the practically identical pattern of the immune response within each group suggests that the different pattern in the two groups must be due to differences between the antigens. The structure of the two polysaccharides and their antigenic determinants is not known in detail.

In the early part of the immunization, the titres of the cross-reacting antibodies were about the same as those of the homologous antibody. Later the homologous antibody showed a much higher activity than the cross-reacting antibody, suggesting that, with continued immunization, antibody-producing clones more specifically adapted to the homologous antigen became predominant.

The reason why antibodies against type 12 remained in the IgM class whereas those against type 13 switched to IgG is obscure. It might be connected with the structure of the antigenic determinant, or of the rest of the molecule, or both, which in some unknown manner inhibits the production of IgG antibodies.

The technical assistance of *Irene Monsen Hortenbach* and *Aud Karin Stendal* is gratefully acknowledged, as well as the valuable help of *Per Torgersen* during the immunization procedure.

REFERENCES

1. *Edwards, P. R. & Fife, M. A.:* Capsule types of *Klebsiella*. J. Infect. Dis. *91:* 92–104, 1952.
2. *Eriksen, J.:* Immunochemical studies on some serological cross-reactions in the *Klebsiella* group. 10. Structure of the capsular polysaccharide of *Klebsiella type 3 (C)*. Acta path. microbiol. scand. *64:* 347–361, 1965.
3. *Eriksen, J.:* Passive hemagglutination test, using erythrocytes sensitized with *Klebsiella* type specific polysaccharides. Acta path. microbiol. scand. Sect. B, *81:* 309–313, 1973.
4. *Eriksen, J. & Haug, R. H.:* Antibodies with restricted electrophoretic heterogeneity in rabbits hyperimmunized with *Klebsiella pneu-*

monia. Acta path. microbiol. scand. Sect. B, *82:* 429–438, 1974.

5. *Eriksen, J., Harboe, M. & Deverill, J.:* A monoclonal IgM with specific antibody reactivity against *Klebsiella* sero-types 12 *and* 13. Acta path. microbiol. scand. Sect. C, *83:* 106–111, 1975.
6. *Harboe, M.:* Unpublished observations.
7. *Harboe, M., Deverill, J. & Eriksen, J.:* Capsular swelling and passive haemagglutination induced by monoclonal IgM reacting with acid polysaccharides of *Klebsiella*. Acta path. microbiol. scand. Sect. C, *83:* 97–105, 1975.
8. *Henriksen, S. D.:* Studies on the *Klebsiella* group. (Kauffmann) I. Serotypes of a collection of strains from human sources and from water. Acta path. microbiol. scand. *34:* 249–258, 1954.
9. *Kabat, E. A. & Mayer, M. M.:* Estimation of protein with biuret and ninhydrin reactions. Experimental Immunochemistry. 2 ed. Charles C. Thomas, Springfield, Illinois, 1967, p. 559.
10. *Kauffmann, F.:* On the serology of the *Klebsiella* group. Acta path. microbiol. scand. *26:* 381–406, 1949.

Acta path. microbiol. scand. Sect. C, *83:* 338–346, 1975

IMMOBILIZING AND CYTOTOXIC SPERM ANTIBODIES IN SERUM AND SEMINAL PLASMA AND THEIR RELATION TO OTHER SPERM ANTIBODIES

S. Husted

Institute of Medical Microbiology, University of Aarhus,
and the University Maternity Hospital, Aarhus, Denmark

Husted, S. Immobilizing and cytotoxic sperm antibodies in serum and seminal plasma their relation to other sperm antibodies. Acta path. microbiol. scand. Sect. C, *83:* 338–346, 1975.

The relation between immobilizing and cytotoxic sperm antibodies and other sperm antibodies (agglutinins and IF-antibodies) in serum and seminal plasma was studied in men belonging in a group of infertile couples. In serum, correlation between the agglutinin titre and the titres of immobilization and cytotoxicity was found to be close, but the titre level of the complement-dependent activities was considerably lower than that of the agglutinins. In seminal plasma, immobilization and cytotoxicity were rarely observed and only in low titres. Absorption of IgG (IgG1, IgG2, IgG4) with protein-A-producing *Staphylococcus aureus* suggested that immobilizing, cytotoxic, and agglutinating antibodies in serum belonged to the IgG class. In seminal plasma, agglutinins seemed mainly to be "non-IgG"—probably IgA. Correlation between occurrence of agglutinating, immobilizing and cytotoxic antibodies in serum on the one hand and low spermcounts or high spermimmotility rate in the ejaculate on the other, was not found.

Key words: Sperm antibodies; immobilizing; cytotoxic; serum; seminal plasma

S. Husted, Institute of Medical Microbiology, University of Aarhus, DK-8000 Aarhus C, Denmark.

Received 11.iv.75 Accepted 10.v.75

Spermatozoal antibodies with complement-dependent immobilizing activity have been demonstrated by various methods in some sperm-agglutinating sera from male partners in infertile couples (*Wilson* 1954, *Rümke & Hellinga* 1959, *Fjällbrant* 1969, *Brogaard Hansen* 1974). Similarly, antibodies with a cytotoxic action in the presence of complement have often been observed by testing male sera containing sperm agglutinins in high titres, but in some cases, the phenomenon has also been observed in sera without agglutinins. Sera containing cytotoxic sperm antibodies have often been found to contain also sperm antibodies, detectable by the indirect immunofluorescent technique (IFT), against the acrosomal region and midpiece of the spermatozoa (*Hamerlynck* 1970). These studies concentrated on only one of the

two complement-dependent activities in male sera, and these studies do consequently not allow a detailed analysis of the relation between sperm immobilization and cytotoxicity.

The aim of the investigation reported here was therefore to study the occurrence of immobilizing and cytotoxic antibodies in serum and seminal plasma from three selected groups of male patients with sperm agglutinins, IF-antibodies, and without these antibodies in serum. This offers the possibility of observing a correlation, if any, between the appearance of complement-dependent activities and other sperm antibodies. The immunoglobulin classes of immobilizing and cytotoxic antibodies and agglutinins in serum and seminal plasma were characterized by an indirect approach as "IgG"—(IgG1, IgG2 and IgG4)—or "non-IgG" by absorption with protein-A-producing *Staphylococcus aureus*.

In animal immunization experiments, *Voisin & Toullet* (1973) found that agglutinating, immobilizing and cytotoxic sperm antibodies seemed directly to induce aspermatogenic orchitis. Therefore, the results obtained by examination of the ejaculates were compared with the antibody findings in the patients.

MATERIAL AND METHODS

The investigation comprised three selected groups of male partners in couples with infertility problems for more than two years.

Group I. Thirty-seven men of an average age of 32.6 years in whom sperm agglutinating antibodies had been found in serum in titres ranging from 4 to 4096.

Group II. Twenty-three men of an average age of 30.0 years in whom IF-antibodies against one or more of the four antigens had been demonstrated in serum in titres ≥10. In none of the cases, sperm agglutinins were present in serum or seminal plasma.

Group III. Onehundred men of an average age of 29.1 years in whom neither sperm agglutinins, nor IF-antibodies were detectable in serum or seminal plasma.

Venous blood was drawn from the patients at the time of the clinical examination, and serum was stored at −20° C until tested. Ejaculates from the men were examined as previously described (*Husted* 1975) and after separation by centrifugation, the seminal plasma samples were also stored at −20° C until tested.

Microtechnique for Detection of Immobilizing and Cytotoxic Sperm Antibodies

The test was carried out in microchambers in microtrays under liquid paraffin as previously described in detail (*Husted & Hjort* 1975, b). In a microchamber, 1 μl test sample and 2 μl sperm suspension in human AB D-negative serum with known complement activity were mixed, and after incubation at 37° C for 2 hours, 1 μl isotonic 0.8 per cent trypan blue was added, followed by incubation for 1 hour to allow staining of the dead cells. Each sample was also tested after addition of a sperm suspension prepared with the same, but previously inactivated, human serum and agglutination patterns could be read in these mixtures. Complement-independent activity would be detected by comparison between the result obtained in this mixture and the test with known negative serum.

The reactions were read under an inverted microscope at 320 ×. The percentages both of immotile and stained spermatozoa in each microchamber were estimated and scored as follows: if less than 25 per cent of the spermatozoa were immotile or stained, 0; if 25–50 per cent were affected, +; 50–75 per cent, ++; more than 75, +++. Only differences of at least two steps in scores between mixtures with and without complement were taken as evidence of complement-dependent activity. The results denoting immobilizing and cytotoxic effect were expressed in titres representing the reciprocal of the highest dilution in which a significant difference was found.

In the preparation of sperm suspensions it is of importance to use a sperm sample of optimal quality with less than 25 per cent immotile spermatozoa.

Dilutions of inactivated serum and seminal plasma in isotonic saline were prepared. Positive and doubtful reactions were retested in threefold serial dilutions starting at undiluted. Absorbed serum samples were tested in threefold dilutions starting at 1:2. Known positive and negative sera were included in each batch.

Sperm-agglutination Test

The gelatin-agglutination test of *Kibrick et al.* (1952) was carried out in a slightly modified form (*Husted & Hjort* 1974). Fresh ejaculates of good quality were used (>60 mill per ml; less than 30 per cent immotile). Equal volumes (0.15 ml) of dilutions of inactivated serum or seminal plasma and sperm suspension (20 mill per ml in Baker's solution with 5 per cent gelatin) were mixed at 37° C, transferred to narrow test tubes (inside diameter 2 mm) and read macroscopically

after incubation at 37° C for 2 hours. All sera and semen samples were screened in dilutions 1:4 and 1:16, and sera with positive or doubtful reactions were retested in fourfold titrations with spermatozoa from two donors. If any difference in titre was found, the lower value was recorded. Known positive and negative sera were included in each batch.

Indirect Immunofluorescent Technique (IFT)

Washed human spermatozoa from an ejaculate of good quality, dried on a slide, and fixed in absolute methanol served as the antigen (*Husted* & *Hjort* 1974). Dilutions of inactivated serum or seminal plasma were applied for 1 hour and FITC-conjugated horse anti-human immunoglobulin (detecting IgG, IgM and IgA) (molar F/P ratio 2.6) for 30 minutes. Each step was followed by washings in phosphate-buffered saline (pH 7.2). After mounting, the reactions were read under a Zeiss fluorescence microscope equipped with interference filter especially adjusted to fluorescein isothiocyanat (*Rygaard* & *Olsen* 1969).

Proper dilutions of human sera containing antibodies against each of the four antigens, negative serum and buffer control were included in each test. The sera were screened in the dilutions 1:10 and 1:30 and the seminal plasma was screened undiluted and 1:4.

Positive and doubtful reactions with the polyvalent conjugate were retested with monospecific FITC-conjugated anti-IgG, anti-IgM and anti-IgA in doubling dilutions, starting with 1:10 in the case of serum and undiluted in the case of seminal plasma in order to detect and quantify antibodies of different immunoglobulin classes. The molar F/P ratios of these conjugates were: anti-IgG, 2.3, anti-IgM, 2.3; anti-IgA, 3.4. The conjugates were used in dilutions corresponding to 1/4–1/8 unit per ml based on chessboard titrations (*Beutner et al.* 1968).

Absorption of IgG by Protein-A-producing Staphylococcus aureus

The ability of some strains of protein-A-producing *Staph aureus* selectively to absorb human IgG molecules (IgG1, IgG2 and IgG4) through binding of the Fc-part of the molecule (*Lind* & *Mansa* 1968, *Kronvall* & *Williams* 1969) was utilized in absorption experiments using serum and seminal plasma samples as previous described (*Husted* & *Hjort* 1975, a). *Staph aureus* strain 4972 was provided by Dr. *I. Lind*, Statens Seruminstitut, Copenhagen.

Harvested staphylococci, suspended in 3 per cent formalin in PBS and left for 30 minutes followed by washings in PBS, were distributed to test tubes to constitute 0.3 ml packed bacteria after centrifugation. 0.3 ml of a 1:2 dilution of inactivated serum or undiluted seminal plasma was added, and the contents were mixed for 30 minutes by slow rotation. After separation of the staphylococci by centrifugations, the test samples were subjected to the Kibrick test in fourfold dilutions starting at 1:4. Samples were tested by the microtechnique in threefold dilutions starting at 1:2 in the case of serum and undiluted in the case of seminal plasma. Unabsorbed, but otherwise similarly treated serum and seminal plasma dilutions from the same sample were tested in the same batch. As an additional precaution, a negative serum was included in each experiment. The concentrations of IgG, IgA and IgM in serum and seminal plasma before and after absorption were determined by the radial immunodiffusion technique using immunodiffusion plates (Tripartigen, LC plates (Behringwerke), respectively). As standard served Standard Human serum (Behringwerke). The diffusion time at room temperature was three days.

RESULTS

I. Immobilizing and Cytotoxic Sperm Antibodies in Serum and Seminal Plasma and Their Relation to Agglutinins and IF-antibodies

It appears from Table 1 that immobilizing and cytotoxic activities were observed only in the test samples containing agglutinating antibodies. In seven of the agglutinating sera showing complement-dependent activity, however, IF-antibodies against various antigens were also present, but these antibodies seemed to be without immobilizing and cytotoxic activity. This appears from the results in group II which was selected in such a manner that sera with antibodies—in titres from 10–40 —against the four most common IF-antigens were present: front part of acrosome, 12; equatorial segment of acrosome, 7; postnuclear cap, 1, main tail piece, 8. Furthermore, the antibodies represented different immunoglobulin classes (front part of acrosome, mainly IgM and in one case IgG; equatorial segment, mostly IgG, but also IgM and IgA; postnuclear cap, IgM; main tail piece, IgG and in one case IgM). In spite of the wide spectrum in antibody specificity and immunoglobulin classes, no immobilizing or cytotoxic activity was observed in this group.

Table 2 shows the occurrence of immo-

TABLE 1. *Occurrence of Complement-dependent Immobilization and Cytotoxicity in Serum and Seminal Plasma in Men in the Three Groups*

Group	Total	Complement-dependent antibodies			
		Serum		Seminal plasma	
		Immobil.	Cytotox.	Immobil.	Cytotox.
I	37*	27	26	4	3
II	23	0	0	0	0
III	100	0	0	0	0

* Seven of these patients had also IF-antibodies in serum.

bilizing and cytotoxic antibodies in serum and seminal plasma from patients in group I. In serum, immobilization and cytotoxicity were detected in 26 of the 37 cases; using undiluted serum, nothing but immobilization was observed in one additional case. The titre of immobilization was in eight cases one threefold titre step higher than that of cytotoxicity, but the latter was not in any case higher than that of immobilization. Furthermore, isolated strong reactions of immobilization or cytotoxicity were not observed.

If the sperm-agglutinin titre in serum is taken into consideration, it appears that immobilization and cytotoxicity were most commonly found in sera with high agglutinin titres as these activities were recorded in 25 of the 27 cases with a titre of ≥ 64, but only in two of the 10 cases with agglutinin titres less than 64. Thus the results in the table indicate a close correlation in serum between the agglutinin titre and the titres of complement-depending activities.

Sperm immobilization and cytotoxicity were observed both if sera causing head-to-head agglutination were used and if sera causing tail-to-tail agglutination were used, which included most of the cases in the series. Head-to-head agglutinates were observed only in three sera with agglutinin titres in the Kibrick test of 4, 16 and 256, respectively, and both immobilization and cytotoxicity occurred in the two strongest sera with titres of 3-3 and 9-9, respectively. Mixed agglutination involving both sperm heads and tails was seen in four cases with agglutinin titres of 4, 4, 4 and 16, but complement-dependent activity was not found in any of the cases.

In seminal plasma, the sperm-agglutinin titres were generally lower than in serum. Sperm agglutinins were found in 29 of the 37 cases. In six cases, the seminal plasma titres were at the same level as the serum titres; in 20 cases, the titres were one fourfold titre step, in 10 cases, two fourfold titre steps and in one case, 4 fourfold titre steps lower than in serum. The agglutination patterns to be observed if seminal plasma was used were only of the tail-to-tail type. In contrast to the findings in serum, immobilizing and cytotoxic sperm antibodies rarely occurred and, if so, in low titres. Immobilization was detected in four samples, including three in which cytotoxicity was also recorded. In these four patients, the serum titres of immobilization and cytotoxicity were relatively high (3–3, 9–3, 9–3, 27–9, respectively) and, at the same time, the sperm-agglutinin titres were high both in serum and seminal plasma (in serum, 256, 256, 1024, 4096; in seminal plasma, 64, 256, 256, 1024).

II. Immunoglobin Classes of Immobilizing, Cytotoxic, and Agglutinating Antibodies in Serum and Seminal Plasma

Table 3 shows the results of absorption by packed formalin-treated protein-A-producing *Staph. aureus* of serum and seminal plasma from nine patients with titres for immobilization and cytotoxicity of 3 or more and also high agglutinin titres in serum. In the serum,

TABLE 2. *Relation between Immobilizing and Cytotoxic Antibodies and Agglutinins in Serum and Seminal Plasma*

	Aggl. titre	No. of patients	Number of patients with Immobilizing antibody titre					Number of patients with Cytotoxic antibody titre				
			neg.	1	3	9	27	neg.	1	3	9	27
Serum	4	5	5	–	–	–	–	5	–	–	–	–
	16	5	3	1	1	–	–	4	–	1	–	–
	64	4	1	1	2	–	–	1	1	2	–	–
	256	16	1	2	7	5	1	1	3	10	2	–
	1024	5	0	–	2	3	–	0	–	3	2	–
	4096	2	0	–	–	–	2	0	–	–	1	–
Total		37	10	4	12	8	3	11	4	16	5	1
Seminal plasma	<4	8	8	–	–	–	–	8	–	–	–	–
	4	3	3	–	–	–	–	3	–	–	–	–
	16	5	5	–	–	–	–	5	–	–	–	–
	64	14	13	1	–	–	–	13	1	–	–	–
	256	6	4	2	–	–	–	5	1	–	–	–
	1024	1	0	–	1	–	–	0	–	1	–	–
Total		37	33	3	1	–	–	34	2	1	–	–

TABLE 3 *Titres of Immobilising, Cytotoxic and Agglutinating Antibodies in Serum and Seminal Plasma before and after Absorption by Staph. aureus*

Serum: Imm. titre U/A	Serum: Cyt titre U/A	Serum: Aggl. titre U/A	Serum: IgG U mg%	Serum: IgG A mg%	Serum: IgM U mg%	Serum: IgM A mg%	Serum: IgA U mg%	Serum: IgA A mg%	Seminal plasma: Imm. titre U/A	Seminal plasma: Cyt. titre U/A	Seminal plasma: Aggl. titre U/A	Seminal plasma: IgG U/A mg%	Seminal plasma: IgM U/A mg%	Seminal plasma: IgA* U/A mg%
3/<2	3/<2	256/4	1210	64	96	88	128	112	1/0	1/0	64/64	6.8/0	0/0	4.8/4.8
3/<2	3/<2	256/<4	1155	60	115	115	204	176	0/0	0/0	16/4	13.3/0	0/0	5.2/5 0
3/<2	3/<2	256/16	1100	48	85	85	152	140	0/0	0/0	64/64	15.6/0	0/0	4.8/4.6
9/<2	3/<2	256/4	1080	56	108	96	180	180	1/0	0/0	256/64	14.2/0	0/0	4.2/4.2
9/2	3/<2	256/4	1250	68	72	72	136	120	0/0	0/0	256/256	11.2/0	0/0	3.6/4.2
27/<2	9/<2	256/<4	1050	88	96	64	168	152	0/0	0/0	256/256	24 0/0	0/0	3.6/3.6
9/<2	9/<2	1024/4	1010	60	106	92	120	120	1/0	1/1	256/64	16.0/0	0/0	4.2/4.0
9/<2	3/<2	1024/4	896	68	72	72	136	96	0/0	0/0	64/64	8.1/0	0/0	3.0/3.0
27/<2	9/<2	4096/16	990	28	60	60	236	236	3/0	3/0	1024/256	6.8/0	0/0	3.0/3.0
Mean concentrations*			1082	60	90	83	162	148				12.9		4.0/4.0

U: Unabsorbed, A: Absorbed.
* The actual concentrations may be between 1 and 3 times as high as those recorded, as seminal plasma may contain secretory IgA (see text).

TABLE 4. *Results of Seminal Examination in the Patients in the Three Groups*

Group	No. of patients	Sperm concentration: Azoo. No.	Azoo. %	Ex.01. No.	Ex.01. %	01. No.	01. %	Norm. No.	Norm. %	Immotile spermatozoa: <40 % No.	<40 % %	40–60 % No.	40–60 % %	>60 % No.	>60 % %
I. + C dep. activity	27	0	–	2	7	4	15	21	78	16	59	7	26	4	15
— C dep. activity	10	1	10	2	20	0	–	7	70	5	56	2	22	2	22
II.	23	3	13	3	13	4	17	13	57	14	70	3	15	3	15
III.	100	8	8	9	9	14	14	69	69	64	70	18	20	10	11

Azoo.: Azoospermia.
Ex.01.: Extreme oligospermia.
01.: Oligospermia.
Norm.: Normospermia.

immobilizing and cytotoxic antibodies were absorbed to such an extent that they were no longer detectable except in one case, and the concentrations of agglutinating antibodies were also strongly reduced, much to the same degree as the serum IgG concentration.

In the seminal plasma, the IgG level was found to be about 1 per cent of the serum level and, after absorption, IgG could not be detected by the radial immunodiffusion technique. IgA was not affected by the absorption. The concentration of IgA in the seminal plasma should, however, be taken with reservation as no distinction could be made between monomeric and secretory IgA by the quantitation technique applied. According to the studies by *Tomasi & Bienenstock* (1968), the values obtained by the technique had to be multiplied by approximately 3 if pure secretory IgA was quantitated, but as both monomeric and secretory IgA are found in seminal plasma, the actual concentrations are between 1 and 3 times as high as those listed in Table 3.

In seminal plasma, immobilizing and cytotoxic antibodies disappeared after absorption, but in contrast to the findings in serum, the agglutinin titres were only slightly affected. It may be noticed that, in three of the four cases in which complement-dependent activity had been detected before absorption, the agglutinin titre was reduced by one fourfold titre step. However, in one of the cases the agglutinin titre was unaffected.

III. Comparison between the Results of the Seminal Evaluation and the Antibody Findings in Serum

Table 4 shows the frequencies of azoospermia, extreme oligospermia (<5 mill spermatozoa per ml), oligospermia (5–20 mill per ml) and normospermia (>20 mill per ml) in the patients in the three groups. Patients in group I are classified into two subgroups: one in which immobilizing and cytotoxic antibodies were present and another in which only agglutinins were found. It appears that no significant difference between the sperm counts in the various groups was revealed. Among the patients with immobilizing and cytotoxic antibodies, no case of azoospermia was recorded and the frequencies of oligo- and normospermia were at the same levels as those observed in patients without demonstrable sperm antibodies.

Furthermore, the presence of the various antibodies in serum does not seem to influence the number of immotile spermatozoa in the ejaculate, as a grouping of the patients, according to percentages of immotile spermatozoa counted after liquefaction of the ejaculate, shows the degrees of immotility to appear at the same frequencies in the three groups.

In the four patients with detectable immobilizing antibodies in seminal plasma, the percentages of immotile spermatozoa after ejaculation were 35, 40, 40 and 50 per cent and, even after 12 hours' storage at 37° C, 10–20 per cent of the spermatozoa in the samples were still motile.

DISCUSSION

The phenomena of complement-dependent sperm immobilization and cytotoxicity were observed only in sera with fairly high titres of sperm agglutinins, whereas sera containing IF-antibodies in relatively high titres or sera without detectable antibodies in all cases showed negative results. This correlation between the appearance of complement-dependent activities and agglutinins would suggest that immobilization, cytotoxicity and sperm agglutination are caused by one and the same antibody, the type of reaction depending on the presence or absence of complement. The antibodies showing complement-dependent activities and the agglutinins are apparently directed against antigens on the surface membranes on the spermatozoa, while IF-antibodies are directed against structures beneath the membrane.

Relatively large differences between the titre levels for agglutination and immobilization and cytotoxicity were found. How-

glutinating sera from men. Acta obstet. gynec. scand. suppl. *36:* 43-50, 1974.
Hamerlynck, J. V. T. H.: Cytotoxic and other auto-antibodies against spermatozoa in relation to infertility in the human male. Thesis. University of Amsterdam. 1970.
Hansen, K. Brogaard: A comparative study of various methods for the detection of spermatozoal antibodies. Acta. obstet. gynec. scand. *53:* 69-76, 1974.
Husted, S. & *Hjort, T.:* Comparison of the occurrence of spermatozoal antibodies in male and female blood donors. Clin. exp. Immunol. *17:* 61-69, 1974.
Husted, S.: Sperm antibodies in men from infertile couples. Int. J. Fertil. 1975. In press.
Husted, S. & *Hjort, T.:* Sperm antibodies in serum and seminal plasma. Int. J. Fertil. 1975 a. In press.
Husted, S. & *Hjort, T.:* Microtechnique for simultaneous determination of immobilizing and cytotoxic sperm antibodies. Clin. exp. Immunol. 1975 b. In press.
Kibrick, S., Belding, D. L. & *Merrill, B.:* Methods for the detection of antibodies against mammalian spermatozoa. II. A gelatin agglutination test. Fertil. and Steril. *3:* 430-438, 1952.
Kronvall, G. & *Williams, R. C.:* Differences in anti-protein A activity among IgG subgroups. J. Immunol. *103:* 828-833, 1969.
Lind, I. & *Mansa, B.:* Further investigation of specific and nonspecific adsorption of serum globulins to *Staphylococcus aureus*. Acta path. microbiol. scand. *73:* 637-645, 1968.
Rygaard, J. & *Olsen, W.:* Interference filters for improved immunofluorescent microscopy. Acta path. microbiol. scand. *76:* 146-148, 1969.
Rümke, P. & *Hellinga, G.:* Autoantibodies against spermatozoa in sterile men. Am. J. Clin. Pathol. *32:* 357-363, 1959.
Tomasi, T. B. & *Bienenstock, J.:* Secretory immunoglobulins. Adv. Immunol. *9:* 1-87, 1968.
Voisin, G. A. & *Toullet, F.:* Auto-immune aspermatogenic orchitis (AIAO) induced by different spermatozoa auto-antigens and different immuno-pathological mechanisms. In: Bratanov, K. (Ed.): Immunology of Reproduction. Sofia, Bulg. Acad. Sci., 1973, p. 57-61.
Wilson, L.: Sperm agglutinins in human semen and blood. Proc. Soc. exp. Biol. (N.Y.) *85:* 652-655, 1954.

Acta path. microbiol. scand. Sect. C, *83:* 347–353, 1975

COMPARISON OF METHODS FOR THE DETECTION OF IMMOBILIZING AND CYTOTOXIC SPERM ANTIBODIES

S. Husted and H. J. Ingerslev

Institute of Medical Microbiology, University of Aarhus, and the University Maternity Hospital, Aarhus, Denmark

Husted, S. & Ingerslev, H. J. Comparison of methods for the detection of immobilizing and cytotoxic sperm antibodies. Acta path. microbiol. scand. Sect. C, *83:* 347–353, 1975.

A sperm-immobilization test, a sperm-cytotoxicity test and a microtechnique for simultaneous detection of immobilizing and cytotoxic sperm antibodies have been compared. The immobilization test and the microtechnique were easy to use. In order to ensure reliable results of the cytotoxicity test, the evaluation of a serum had to be based on the mean spermotoxic index (STI) of three experiments. The cytotoxicity test was therefore found to be more time-consuming and less suitable for large-scale examinations. Among 30 sera from men with sperm agglutinins, all sera with titres >64 yielded positive results by all three techniques, whereas minor differences were found in sera with lower agglutinin titres. A comparison of the tests showed that, except for one case, cytotoxic activity was observed only in sera which also had immobilizing activity and, using the microtechnique, this was observed without exception. A total of 103 sera without agglutinins revealed negative results by the three techniques. Titration of positive sera in the immobilization test and in the microtechnique using guinea-pig and human serum, respectively, as the source of complement, showed identical results if the actual dilution of test serum in the test mixture was taken into account.

Key words: Sperm antibodies; immobilizing; cytotoxic; detection methods; comparison.

S. Husted, Institute of Medical Microbiology, University of Aarhus, DK-8000 Aarhus C, Denmark.

Received 10.v.75 Accepted 10.v.75

A recently described microtechnique, elaborated according to the principles used for tissue typing of lymphocytes, has made it possible simultaneously to study complement-dependent immobilizing and cytotoxic activities in sera. In sera from male partners in infertile couples, the simultaneous occurrence of sperm agglutinins in sera causing immobilization and cytotoxicity suggested that these phenomena were complement-dependent effects of the sperm agglutinins (*Husted* 1975). Previously, the occurrence of immobilizing antibodies has been studied most extensively in female partners in infertile couples (*Isojima et al.* 1968; *Isojima et al.* 1972), but such antibodies have also been found in men (*Wilson* 1954, *Rümke & Hellinga* 1959, *Fjällbrant* 1969). In sera from men, the complement-dependent cytotoxic action on spermatozoa has been studied by *Hamerlynck & Rümke* (1968), *Hamerlynck* (1970), *Hamerlynck & Rümke* (1974).

The aim of this investigation was to evaluate the method for sperm immobilization, a slightly modified method for cytotoxicity and

the microtechnique, and to compare the results obtained by testing sera from a group of men with sperm agglutinating antibodies and a group of women without detectable agglutinating antibodies. In this way it is also possible to study the results of all three tests in relation to the occurrence of sperm agglutinins.

MATERIAL AND METHODS

The investigation included patients in two selected groups of couples with infertility problems for more than two years.

Group I: Thirty men of an average age of 31.8 years in whom sperm-agglutinating antibodies had been found in serum in titres ranging from 4 to 4096.

Group II: One hundred and three women of an average age of 30.2 years in whom agglutinating antibodies in serum were not detectable by the gelatin-agglutination test (Kibrick test).

Venous blood was drawn from the patients at the time of the clinical examination and the serum was stored at –20° C until tested.

Sperm-immobilization Test

The immobilization test introduced by *Isojima et al* (1968) was carried out in a slightly modified form. Amounts of 0.25 ml inactivated test serum, 0 025 ml sperm suspension and 0.05 ml complement-containing guinea-pig serum were mixed in test tubes, incubated at 37° C for 60 minutes, followed by mixing for a short time in order to avoid sedimentation of agglutinated spermatozoa. One drop of the suspension was then transferred to a slide and the percentage of motile spermatozoa (T) was determined by counting about 100 spermatozoa under the phase-contrast microscope at 400 ×. Similarly, in each test, the motility percentage was determined by a serum known to be negative (C). The calculated sperm-immobilizing value—SIV = C/T—was considered to be significant if it was higher than 2.

The sperm suspension was prepared from a fresh ejaculate of good quality (≥60 mill spermatozoa per ml and less than 30 per cent immotile). After centrifugation for 5–10 minutes at 800–1000 rev/min, the supernatant was pipetted off and discarded and the spermatozoa were resuspended to a concentration of 60 mill per ml in inactivated normal human AB Rh D-neg. serum.

Guinea-pig sera were commonly found to exert a sperm-immobilizing effect. Sera without such activity were selected for the experiments. Among 15 guinea pigs, only serum from three was found usable in the test. The activity of the complement was controlled in a haemolytic system.

The inactivated test sera (30 minutes at 56° C) were screened undiluted. In addition to the negative serum previously mentioned, a positive serum was included in each batch. The experiments were discarded if the motility percentage C differed significantly from that of the untouched sperm suspension stored under the same conditions as the other tubes.

Sperm-cytotoxicity Test

The test introduced by *Hamerlynck & Rümke* (1968), *Hamerlynck* (1970), *Hamerlynck & Rümke* (1974, 1975) was performed in a slightly modified way. One drop of inactivated test serum, one drop of sperm suspension and two drops of human AB Rh D-neg. serum containing complement were mixed in a test tube. After incubation for one hour at 37° C, a further two drops of fresh AB Rh D-neg. serum were added and this mixture was incubated for another hour. Then two drops of dye (0.67 per cent eosin and 10 per cent nigrosin in PBS) were added and, after gentle mixing, incubation for 20 minutes at room temperature followed. A smear was then made on a slide, dried in an air current, and mounted in plastic grease. By way of control, each serum was tested in the same way the only change being that the added AB Rh D-neg. serum had previously been inactivated. The number of spermatozoa in the control smear necessary for counting 100 unstained was determined (100+d) under the bright-field microscope. Among 100+d spermatozoa in the test smear, the number of unstained spermatozoa, ×, was recorded and the spermatoxic index (STI) was calculated as 100-×. STI was considered significant if it exceeded 25, doubtful between 16 and 25, and negative if it was below 16. If 100+d exceeded 250, indicating more than 60 per cent killed spermatozoa in the control, the experiment was excluded.

The sperm suspension was prepared from a fresh ejaculate of good quality, washed once in PBS (pH 7.2) by slow centrifugation for 15 minutes and resuspended in PBS to a concentration of 100 mill per ml. The human AB Rh D-neg. serum did not contain sperm antibodies and was of known complement activity. The serum was stored in small test tubes at –70° C until used.

The test sera were screened undiluted. Sera with positive or doubtful reactions were retested. Sera known to be positive and negative were included in each batch.

Microtechnique for Detection of Immobilizing and Cytotoxic Sperm Antibodies

The test was carried out in microchambers in microtrays under liquid paraffin as previously de-

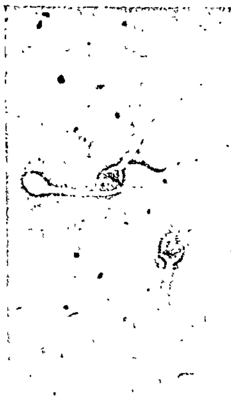


Fig 1. Sperm cytotoxicity test: One unstained and two stained spermatozoa. Approximately × 2250.

scribed in detail (*Husted & Hjort* 1975). In a microchamber, 1 μl of test sample and 2 μl of sperm suspension in human AB D-neg. serum with known complement activity were mixed and incubated at 37° C for two hours, upon which 1 μl isotonic 0.8 per cent trypan blue was added. This mixture was incubated for one hour to allow staining of dead cells. Each sample was tested in the same way except that previously inactivated AB Rh D-neg. serum was used for the sperm suspension. Comparison of this sample and the negative control would disclose complement-independent activity.

The reactions were read under the inverted microscope and the percentages both of immotile and stained spermatozoa in each microchamber were estimated and scored as follows: if less than 25 per cent affected: 0; 25–50 per cent. +; 50–75 per cent· + +; more than 75 per cent: + + +. Differences of at least two steps in scores between mixtures with and without complement were considered significant in that they showed complement-dependent activity. The results denoting immobilization and cytotoxicity were expressed in titres representing the reciprocal of the highest dilution which was found to differ significantly.

The sperm suspensions were prepared from a fresh ejaculate (about 100 mill spermatozoa per ml and less than 25 per cent immotile). Dilutions of previously inactivated serum in isotonic saline were prepared. Sera giving positive and doubtful reactions were retested in threefold serial dilutions, starting at undiluted. Positive and negative sera were included in each batch.

Sperm-agglutination Test

The gelatin-agglutination test introduced by *Kibrick et al.* (1952) was carried out as previously described (*Husted & Hjort* 1974). All sera were screened in the dilutions 1:4 and 1:16, and sera with positive or doubtful reactions were retested in fourfold titrations. Sera known to be positive and negative were included in each batch.

RESULTS

I. Methodological Modifications

Washing procedures have been found to cause an increase in the immotility rate and, in some cases, even to alter the surface membranes of the spermatozoa. Therefore, in the sperm-immobilization test, the sperm suspension was prepared exclusively by centrifugation of the ejaculate and resuspension of the sedimented spermatozoa in previously inactivated normal human AB D-neg. serum which was found to be a suitable medium for spermatozoa.

By performance of the sperm-cytotoxicity test according to the modification suggested by *Hamerlynck & Rümke* (1974, 1975) we found, like the latter authors, that 40–50 per cent of the experiments had to be discarded because of non-specific killing of the spermatozoa during the experiment. Examination of the different steps of the original procedure showed that the number of non-specifically killed spermatozoa was greatly increased during the final centrifugation for 30 minutes. In the original procedure this centrifugation is needed to concentrate the spermatozoa because a fairly large volume of staining mixture (18 drops) is added. However, by addition of only two drops of dye, the dilution of the test mixture was reduced to such an extent that centrifugation could be omitted. This could be done without altering significantly the intensity of the staining of killed

TABLE 1. *Comparison of the Results Obtained when a Negative Serum is Tested 20 Times in One Experiment by the Three Methods*

Method	Results					
	Negative		Doubtful		Positive	
	No.	%	No.	%	No.	%
Sperm-immobilization test	20	100	–	–	–	
Sperm-cytotoxicity test	17	85	2	10	1	5
Microtechnique:						
Immobilization	20	100	–		–	
Cytotoxicity	20	100	–		–	

spermatozoa or the contrast between unstained spermatozoa and the dark background (Fig. 1).

The rate of successful experiments was considerably increased by this modification. However, the number of dead spermatozoa still fluctuated to some extent. In a given experiment, some of the sera known to be negative could suddenly show doubtful or weakly positive STI values. According to the original method, the STI showed the difference between reactions with a negative serum and a test serum. In order to eliminate the influence of non-specific factors we based the STI of a serum on the countings from a reaction with complement and a similar reaction in which the complement source had previously been inactivated. As negative control sera were included in each batch, this procedure made it possible to distinguish between complement-dependent and complement-independent cytotoxicity.

II. Comparison of the Tests

a. Repeatability and reliability of the tests. Table 1 shows the repeatability of the methods, when testing one serum known to be negative 20 times in the same experiment. By the sperm-immobilization test, the SIV value was 1.14 with a variation coefficient u = 0.23. By the sperm-cytotoxicity test, the serum was negative in 17 cases, but doubtfully positive in two cases and positive in one case. The mean STI value was 2.60 and the variation coefficient u = 15.65. Thus, by tests of unknown sera in only one experiment, a certain percentage would show "false positive" results because of the wide variation in the test. Using the microtechnique, the serum was found to be without immobilizing or cytotoxic activity in all cases.

The reliability of the tests was studied by means of a serum known to be negative which was tested in a series of 12 experiments, using spermatozoa from different donors. By the immobilization test and the microtechnique, the serum was found to be negative in all experiments. By the cytotoxicity test the serum was negative only in 10 cases and positive in two cases (16.6 per cent). As expected, the test-to-test variation was wider than that between the test mixtures used in the same experiment.

In order to minimize the possibility that a serum was found to be "false positive" because of the variability we decided that the evaluation of a serum should be based on the mean STI of several experiments—in this study three.

b. Results obtained in sera from groups I and II. Table 2 shows a comparison between the results obtained by the different techniques and sera from men in group I—30 men with sperm agglutinins demonstrable in serum —and from women in group II—103 women without agglutinins in serum.

In group I it appears that immobilization and cytotoxicity were recorded more often by the sperm-immobilization test and by the cytotoxicity test, respectively, than by the microtechnique showing at the same time both phenomena (24 versus 21 cases) and

TABLE 2. *Results in Sera from Groups I and II*

Method	Group	Agglutinin titre	No. of sera	Immobilization Neg.	Immobilization Pos.	Cytotoxicity Neg.	Cytotoxicity Pos.
Sperm-immobilization test	I	>64	15	0	15		
		=64	7	0	7		
		≥4, <64	8	6	2		
		Total	30	6	24		
	II	<4	103	103	0		
Sperm-cytotoxicity test	I	>64	15			0	15
		=64	7			2	5
		≥4, <64	8			6	2
		Total	30			8	22
	II	<4	103			103	0
Microtechnique for immobilization and cytotoxicity	I	>64	15	0	15	0	15
		=64	7	2	5	4	3
		≥4, <64	8	7	1	8	0
		Total	30	9	21	12	18
	II	<4	103	103	0	103	0

(22 versus 18 cases), respectively. This suggests a different sensitivity of the tests. If the agglutinin titres of the sera are taken into account, no differences between the results were found as all sera with agglutinin titres >64 were positive in all three tests. The minor differences were thus detected when sera with agglutinin titres of 64 or less were used.

The three sera revealing positive results by the sperm-immobilization test (SIV values: 20, 2.9, 2.3), but not by the microtechnique, had agglutinin titres of 64, 64 and 16, respectively, and were also found to be negative by the cytotoxicity test.

The agglutinin titres of the four sera found to be positive by the cytotoxicity test with STI of 46, 53, 62 and 35, were 64, 64, 16 and 16, respectively. By the microtechnique, cytotoxic activity was not observed in these sera, whereas immobilization occurred in three of the cases. In the last case with an average STI of 62 (based on three experiments with STI: 96, 48, 42) and agglutinin titre of 16, both the immobilization test and the microtechnique showed negative results.

All the non-agglutinating sera from group II were negative by all three techniques.

A comparison of the results obtained by the immobilization test and the cytotoxicity test shows, with one exception, that cytotoxicity appeared only in sera with immobilizing activity, whereas immobilization in some cases can be observed only in low titres in sera with lower agglutinin titres (≤64). This is in agreement with the experience gained from the microtechnique, in which cytotoxicity appeared only in sera with immobilizing activity.

c. Titration of positive sera in the immobilization tests. In order to analyse the sensitivity of the immobilization tests, a further 12 positive sera were titrated in threefold dilutions; the results are shown in Fig. 2. It appears that, using the sperm-immobilization test, the titres were found to be one or two threefold titre steps higher than those revealed by the microtechnique, but in ord-

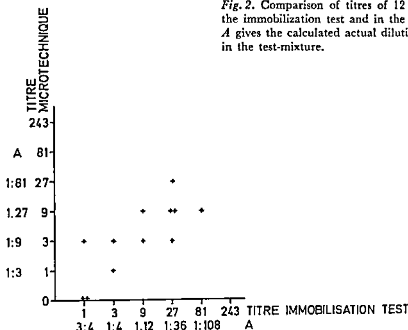


Fig. 2. Comparison of titres of 12 positive sera in the immobilization test and in the microtechnique. *A* gives the calculated actual dilution of the serum in the test-mixture.

to compare the tests it is necessary to take the actual dilution of the sample in the test mixture into consideration. In the sperm-immobilization test used in the present study, the sample to be tested is diluted only to about 3:4, whereas in the microtechnique the serum is tested in the dilution 1:3 because of the large volume of complement. It is seen from the figure that the sensitivity in the two systems are roughly equal and, except for one case, the results differ by only one threefold titre step. The findings in sera revealing positive results by the immobilization test, but not by the microtechnique, may thus be explained solely by the higher serum concentration used in the former technique.

DISCUSSION

The results obtained by the modifications of the sperm-immobilization test and the sperm-cytotoxicity test as well as by the microtechnique for the detection of immobilizing and cytotoxic sperm antibodies showed good agreement. Immobili-·ion and cytotoxicity occurred only in sera ·h sperm-agglutinating activity, suggestin in accordance with previous studies (*Hus l* 1975)—that the two phenomena were complement-dependent effects of sperm-agglutinating antibodies. Some sera with low titres showed only immobilizing activity. In a few cases, the titres recorded for immobilization in the microtechnique were one titre step higher than those recorded for cytotoxicity—but never *vice versa*. Presumably, the staining of the immotile spermatozoa does not occur instantly, but to some degree in proportion to the extent of the lesion of the cell. This would imply that tests detecting immobilization are to be preferred in the *in vitro* study of weak type-II hypersensibility reactions to spermatozoa.

The immobilization test and the microtechnique were both easy to perform, and reliable. One of the major differences between the two tests is the source of complement. The results of the titration experiments suggest that it is not of major importance whether guinea-pig or human complement is used, if only there is a surplus of complement present in the test mixtures. The advantage of using the stronger guinea-pig complement in the sperm-immobilization test is that it allows addition of only a small volume of complement and thus, the original serum

dilution is only slightly influenced. Consequently, in this test the reaction can be observed in an almost undiluted test sample. On the other hand, guinea-pig sera often exert a non-specific immobilizing action on human spermatozoa. Accordingly, selection of guinea-pig sera without the slightest immobilizing activity may present difficulties.

The SIV—the percentage of motile spermatozoa in the negative control divided by the percentage of motile spermatozoa in the test mixture—was in this study found to be inappropriate for quantitation, especially if relatively strong sera are to be tested, as it obviously does not represent a linear, but a hyperbolic function. Consequently, we preferred titration of positive samples and expression in titres representing the reciprocal of the highest dilution revealing a positive reaction. The exact counting used in the immobilization test may give rise to detection of weak reactions that were estimated as negative in the microtechnique, but it renders the technique less suitable for more comprehensive screening experiments. However, the results obtained and the titration experiments in this study do not show any major discrepancy between the tests.

The results obtained by the modified sperm-cytotoxicity test were found mainly to be in agreement with those recorded by the microtechnique. In order to minimize the possibility of "false positive and negative" results it was considered necessary to retest each serum in three experiments and to use the mean STI for evaluation of the serum. This rendered the method very time-consuming and unsuitable for large-scale examinations, even though the smears could be stored for later counting and evaluation.

The detection of cytotoxic activity in three sera—with immobilizing and weak agglutinating activity—at the same time evaluated as negative by the microtechnique, would suggest that counting makes this technique more sensitive than the microtechnique. On the other hand, by this technique, cytotoxicity was rather surprisingly, found in one serum in which immobilizing activity was not revealed by the other techniques.

This investigation was supported by the *Danish Medical Research Council* (Grant No. 512-4200) and the *Danish Foundation for the Advancement of Medical Science* (Grant No. 27/74).

REFERENCES

Fjällbrant, B.: Studies on sera from men with sperm antibodies. Acta obstet. gynec. Scand. *48:* 131–146, 1969.

Hamerlynck, J. & Rümke, P.: A test for the detection of cytotoxic antibodies to spermatozoa in man. J. Reprod. Fertil. *17:* 191–194, 1968.

Hamerlynck, J. V. T. H.: Cytotoxic and other auto-antibodies against spermatozoa in relation to infertility in the human male. Thesis. University of Amsterdam. 1970.

Hamerlynck, J. & Rümke, P.: Spermocytotoxicity test. In WHO Rapport: Techniques for detection of iso- and auto-antibodies to human spermatozoa. In press. 1975.

Hamerlynck, J. & Rümke, P.: Paper presented at WHO workshop on "Techniques for detection of iso- and auto-antibodies to human spermatozoa", Aarhus July 1974.

Husted, S. & Hjort, T.: Comparison of the occurrence of spermatozoal antibodies in male and female blood donors. Clin. exp. Immunol. *17:* 61–69, 1974.

Husted, S. & Hjort, T.: Microtechnique for simultaneous determination of immobilizing and cytotoxic sperm antibodies. Clin. exp. Immunol. 1975 (in press).

Husted, S.: Immobilizing and cytotoxic sperm antibodies in serum and seminal plasma and their relation to other sperm antibodies. Acta path. microbiol. scand. Sect. C, *83:* 338–346, 1975.

Isojima, S., Li, T. S. & Ashitaka, Y.: Immunologic analysis of sperm-immobilizing factor found in sera of women with unexplained sterility. Amer. J. Obstet. Gynec. *101:* 677–683, 1968.

Isojima, S., Tsuchiya, K., Koyama, K., Tanaka, C., Naka, O. & Adachi, H.: Further studies on sperm-immobilizing antibody found in sera of unexplained cases of sterility in women. Amer. J. Obstet. Gynec. *112:* 199–207, 1972.

Kibrick, S., Belding, D. L. & Merrill, B.: Methods for the detection of antibodies against mammalian spermatozoa. II. A gelatin agglutination test. Fertil. and Steril. *3:* 430–438, 1952.

Rümke, P. & Hellinga, G.: Antibodies against spermatozoa in sterile men. Amer. J. clin. Path. *32:* 357–363, 1959.

Wilson, L.: Sperm agglutinins in human semen and blood. Proc. Soc. exp. Biol. (N.Y.) *85:* 652–655, 1954.

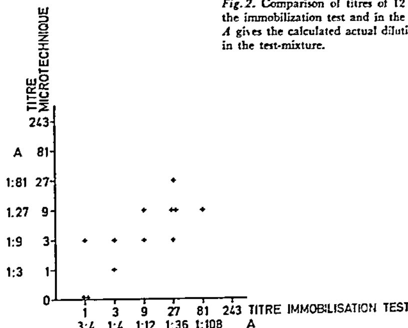


Fig. 2. Comparison of titres of 12 positive sera in the immobilization test and in the microtechnique. *A* gives the calculated actual dilution of the serum in the test-mixture.

to compare the tests it is necessary to take the actual dilution of the sample in the test mixture into consideration. In the sperm-immobilization test used in the present study, the sample to be tested is diluted only to about 3:4, whereas in the microtechnique the serum is tested in the dilution 1:3 because of the large volume of complement. It is seen from the figure that the sensitivity in the two systems are roughly equal and, except for one case, the results differ by only one threefold titre step. The findings in sera revealing positive results by the immobilization test, but not by the microtechnique, may thus be explained solely by the higher serum concentration used in the former technique.

DISCUSSION

The results obtained by the modifications of the sperm-immobilization test and the sperm-cytotoxicity test as well as by the microtechnique for the de[illegible]ction of immobilizing and cytotoxic sperm [illegible]ibodies showed good agreement. Immobi[illegible]on and cytotoxicity occurred only in sera [illegible]h sperm-agglutinating activity, suggestin[illegible]n accordance with previous studies (*Hu*[illegible] 1975)—that the two phenomena were complement-dependent effects of sperm-agglutinating antibodies. Some sera with low titres showed only immobilizing activity. In a few cases, the titres recorded for immobilization in the microtechnique were one titre step higher than those recorded for cytotoxicity—but never *vice versa.* Presumably, the staining of the immotile spermatozoa does not occur instantly, but to some degree in proportion to the extent of the lesion of the cell. This would imply that tests detecting immobilization are to be preferred in the *in vitro* study of weak type-II hypersensibility reactions to spermatozoa.

The immobilization test and the microtechnique were both easy to perform, and reliable. One of the major differences between the two tests is the source of complement. The results of the titration experiments suggest that it is not of major importance whether guinea-pig or human complement is used, if only there is a surplus of complement present in the test mixtures. The advantage of using the stronger guinea-pig complement in the sperm-immobilization test is that it allows addition of only a small volume of complement and thus, the original serum

dilution is only slightly influenced. Consequently, in this test the reaction can be observed in an almost undiluted test sample. On the other hand, guinea-pig sera often exert a non-specific immobilizing action on human spermatozoa. Accordingly, selection of guinea-pig sera without the slightest immobilizing activity may present difficulties.

The SIV—the percentage of motile spermatozoa in the negative control divided by the percentage of motile spermatozoa in the test mixture—was in this study found to be inappropriate for quantitation, especially if relatively strong sera are to be tested, as it obviously does not represent a linear, but a hyperbolic function. Consequently, we preferred titration of positive samples and expression in titres representing the reciprocal of the highest dilution revealing a positive reaction. The exact counting used in the immobilization test may give rise to detection of weak reactions that were estimated as negative in the microtechnique, but it renders the technique less suitable for more comprehensive screening experiments. However, the results obtained and the titration experiments in this study do not show any major discrepancy between the tests.

The results obtained by the modified sperm-cytotoxicity test were found mainly to be in agreement with those recorded by the microtechnique. In order to minimize the possibility of "false positive and negative" results it was considered necessary to retest each serum in three experiments and to use the mean STI for evaluation of the serum. This rendered the method very time-consuming and unsuitable for large-scale examinations, even though the smears could be stored for later counting and evaluation.

The detection of cytotoxic activity in three sera—with immobilizing and weak agglutinating activity—at the same time evaluated as negative by the microtechnique, would suggest that counting makes this technique more sensitive than the microtechnique. On the other hand, by this technique, cytotoxicity was rather surprisingly, found in one serum in which immobilizing activity was not revealed by the other techniques.

This investigation was supported by the *Danish Medical Research Council* (Grant No. 512-4200) and the *Danish Foundation for the Advancement of Medical Science* (Grant No. 27/74).

REFERENCES

Fjällbrant, B.: Studies on sera from men with sperm antibodies. Acta obstet. gynec. Scand. *48:* 131–146, 1969.

Hamerlynck, J. & Rümke, P.: A test for the detection of cytotoxic antibodies to spermatozoa in man. J. Reprod. Fertil. *17:* 191–194, 1968.

Hamerlynck, J. V. T. H.: Cytotoxic and other auto-antibodies against spermatozoa in relation to infertility in the human male. Thesis. University of Amsterdam. 1970.

Hamerlynck, J. & Rümke, P.: Spermocytotoxicity test. In WHO Rapport: Techniques for detection of iso- and auto-antibodies to human spermatozoa. In press. 1975.

Hamerlynck, J. & Rümke, P.: Paper presented at WHO workshop on "Techniques for detection of iso- and auto-antibodies to human spermatozoa", Aarhus July 1974.

Husted, S. & Hjort, T.: Comparison of the occurrence of spermatozoal antibodies in male and female blood donors. Clin. exp. Immunol. *17:* 61–69, 1974.

Husted, S. & Hjort, T.: Microtechnique for simultaneous determination of immobilizing and cytotoxic sperm antibodies. Clin. exp. Immunol. 1975 (in press).

Husted, S.: Immobilizing and cytotoxic sperm antibodies in serum and seminal plasma and their relation to other sperm antibodies. Acta path. microbiol. scand. Sect. C, *83:* 338–346, 1975.

Isojima, S., Li, T. S. & Ashitaka, Y.: Immunologic analysis of sperm-immobilizing factor found in sera of women with unexplained sterility. Amer. J. Obstet. Gynec. *101:* 677–683, 1968.

Isojima, S., Tsuchiya, K., Koyama, K., Tanaka, C., Naka, O. & Adachi, H.: Further studies on sperm-immobilizing antibody found in sera of unexplained cases of sterility in women. Amer. J. Obstet. Gynec. *112:* 199–207, 1972.

Kibrick, S., Belding, D. L. & Merrill, B.: Methods for the detection of antibodies against mammalian spermatozoa. II. A gelatin agglutination test. Fertil. and Steril. *3:* 430–438, 1952.

Rümke, P. & Hellinga, G.: Antibodies against spermatozoa in sterile men. Amer. J. clin. Path. *32:* 357–363, 1959.

Wilson, L.: Sperm agglutinins in human semen and blood. Proc. Soc. exp. Biol. (N.Y.) *85:* 652–655, 1954.

Acta path. microbiol. scand. Sect. C, *83:* 354–364, 1975

CIRCULATING IMMUNE COMPLEXES INVOLVING GRANULOCYTE-SPECIFIC ANTINUCLEAR FACTORS IN FELTY'S SYNDROME AND RHEUMATOID ARTHRITIS

ALLAN WIIK

Immunological Laboratory, University Clinic for Infectious Diseases and Department of Clinical Chemistry, Blegdamshospitalet, Copenhagen, Denmark

Wiik, A. Circulating immune complexes involving granulocyte-specific antinuclear factors in Felty's syndrome and rheumatoid arthritis. Acta path. microbiol. scand. Sect. C, *83:* 354–364, 1975.

This study was designed to look for participation of granulocyte-specific antinuclear factors in circulating immune complexes in rheumatoid arthritis and related conditions. Sera from 20 rheumatoid arthritis patients, 13 of whom displayed the classical Felty's syndrome and 3 showed neutropenia, were fractionated on a Sephadex G-200 column at neutral pH. Two sera from patients with neutropenia, probably of an auto-immune type, were included as well as 3 osteoarthrosis sera and 5 normal control sera. Each fraction was analysed for presence of immunoglobulins G, M and A, antiglobulins, granulocyte-specific and organ-nonspecific antinuclear factors and heterophilic antibodies to rabbit red cells. At neutral pH, macromolecular and intermediate size IgG aggregates were found in most rheumatoid sera but in none of the control sera. The aggregates comprised granulocyte-specific and sometimes organ-nonspecific antinuclear factors of the IgG and often of the IgA classes. Felty patient sera were extraordinarily rich in aggregates containing considerable amounts of IgG granulocyte-specific antinuclear factors. The aggregates usually were dissociated at pH 4.5. IgG and IgA rabbit red cell antibodies mostly appeared in unaggregated form only. Macromolecular IgM antinuclear factors were found in all patient and control sera, but in addition, 80 per cent of the rheumatoid sera contained low molecular size IgM antinuclear factors, probably representing products of local inflammatory processes. The low molecular size IgM antibody activity frequently disappeared after fractionation at acid pH, possibly indicating pronounced sensitivity of these molecules to low pH. Complement-fixing properties of the granulocyte-specific antinuclear factors seemed to be linked to IgG antibodies. IgM rabbit red cell antibodies were solely of the 19S variety except in two cases of Felty's syndrome. Besides macromolecular antiglobulins, many rheumatoid sera contained low molecular size antiglobulins. The data indicate that granulocyte-specific antinuclear factors are regularly involved in circulating immune complexes in rheumatoid arthritis and thus may contribute to the systemic manifestations which are assumed to be caused by such complexes. In cases of neutropenic rheumatoid arthritis, granulocyte-specific antinuclear factors possessing complement-fixing abilities, may augment the phlogogenic effect of the immune complexes.

Key words: Felty's syndrome; rheumatoid arthritis; circulating immune complexes; granulocyte-specific antinuclear factor; 7S IgM.

Allan Wiik, Immunological Laboratory, University Clinic for Infectious Diseases, Blegdamshospitalet, Blegdamsvej 3, DK-200 Copenhagen N, Denmark.

Received 18.iv.75 Accepted 18.iv.75

Rheumatoid arthritis (RA) is predominantly an articular disease manifesting polyarticular chronic synovitis. Extra-articular manifestations, however, are common and should be regarded as typical of the disease (7, 9). Rheumatoid lung disease, pleurisy, pericarditis, polyneuropathy and nodules are some of the outward clinical features of a vasculitis which is considered to be caused by circulating immune complexes (6, 7, 10). Lymphadenopathy and splenomegaly may be the results of a hyperfunctioning immune apparatus and a hyperplastic reticuloendothelial system. Neutropenia is probably the outcome of a rapid removal of neutrophils constantly trying to minimize the level of circulating immune complexes by phagocytosis (11).

This work will focus on the existence of immune complexes in serum of RA patients, paying special attention to some specific antibodies participating in the complexes.

MATERIALS AND METHODS

Sera were obtained from 13 patients with a classical Felty's syndrome, 3 patients with neutropenia in relation to RA, 4 patients with RA in whom numbers of neutrophils in the blood were normal, and 2 patients who showed neutropenia, recurrent fever, myalgias, arthralgias and constantly positive tests for antiglobulins though absence of signs of chronic arthritis. The whole group of patients mentioned is henceforth referred to as the rheumatoid group. All RA patients satisfied the criteria for classical or definite RA (16). Sera from 3 patients with osteo-arthrosis and 5 healthy blood donors served as controls. All sera were stored at -20° C for a period not exceeding a few months until gel filtration was undertaken.

Gel filtration studies. 3 ml of serum was fractionated by gel filtration on Sephadex G-200 (Pharmacia, Uppsala) using a column of 2.5 × 95 cm and a Tris-HCl elution buffer pH 7.35 containing 0.02 M sodium azide, I = 0.154. A constant upward flow was secured by means of a peristaltic pump (Vario perpex, LKB Instruments, Stockholm). The elution flow rate was 8 ml/hour. 8 ml fractions were collected employing a fraction collector (Ultrorac, LKB Instruments, Stockholm). The 280 nm optical density prophile of the eluate was continuously recorded by a Uvicord spectrophotometer, model II (LKB Instruments, Stockholm). All the sera were studied at neutral pH. After equilibration of the same column with acetate buffer pH 4.50, I = 0.154, 16 of the sera (see Table 2) were fractionated on the column using otherwise identical conditions. All fractions were subsequently neutralized by NaOH immediately after the filtration, the final pH of the fractions being 7.3 ± 0.2.

Immunochemical studies. Occurrence of IgG, IgA and IgM in the individual fractions was studied by double immunodiffusion in 1 per cent agar gel containing phosphate buffered saline pH 7.2 (PBS). IgG fractions of rabbit antisera specific for human γ, μ and α chains were employed for the identification (Dakopatts, Copenhagen). The specificity of the antisera was secured by crossed immunoelectrophoresis (13).

Serological studies. Whole sera and serum fractions were investigated for the occurrence of granulocyte-specific antinuclear factors (GS-ANF) and organ-nonspecific antinuclear factors (ON-ANF) belonging to the three main immunoglobulin classes by indirect immunofluorescence using fluorescein-labelled IgG fractions of rabbit antisera specific for human γ, μ and α chains (Dakopatts, Copenhagen). For demonstration of complement-fixing properties of the specific antibodies, the immunofluorescence anticomplement technique was employed using a conjugate specific for human C3 (Dakopatts, Copenhagen) (23). All details concerning the technique, the characteristics of the conjugates and the fluorescence microscope have been reported earlier (22, 23, 24).

Antiglobulins showing specificity for determinants on homologous IgG were demonstrated by the latex fixation slide test (Hyland, Brussels).

Presence of IgG aggregates in the whole sera was studied by precipitation with monoclonal IgM rheumatoid factor in 0.5 agar gel (26). The isolated IgM fraction of a serum from an RA patient with an IgM kappa-M-component having extremely high rheumatoid factor activity was used (kindly donated by *Birger Jensen*, the Copenhagen County Hospital in Gentofte).

Heterophilic antibodies against rabbit red cells (RRC-Ab), one out of many examples of naturally ocurring antibody specificities found in all normal human sera (19), were demonstrated by direct haemagglutination. Rabbit red cells in Alsever's solution (Statens Seruminstitut, Copenhagen) were washed 4 times in PBS. The cells were resuspended in PBS at a concentration of 1 per cent (v/v). 200 μl of fraction and 200 μl suspension were mixed in small glass tubes which were then incubated for one hour at +37° C and finally for 18 hours at +4° C. Agglutination was read in indirect light by gentle tapping on the tubes. Positive reactions were primarily seen in fractions containing IgM agglutinins. In order to study the immunoglobulin class nature of the heterophilic antibodies,

TABLE 1. *Summary of Essential Physical, Immunochemical and Serological Data Obtained from Gel Filtration Studies of Sera at Neutral pH*

	Felty's syndrome	RA with neutropenia	RA without neutropenia	Neutropenia without RA	Osteo-arthrosis	Normal sera
Number of sera	13	3	4	2	3	5
Intermediate size complexes*	4	0	0	0	0	0
IgG in peak I	11	2	1	2	0	0
IgM in peak II	0	0	0	0	0	0
IgA in peak I	8	2	4	2	0	2
IgG ANF in peak I	13 (11 GS, 2 ON)	3 (1 GS, 2 ON)	3 (1 GS, 2 ON)	2 (1 GS, 1 ON)	0 (3)§	0 (5)
IgM ANF in peak II	11 (7 GS, 4 ON)	2 (0 GS, 2 ON)	3 (1 GS, 2 ON)	2 (1 GS, 1 ON)	0	0
IgA ANF in peak I	10 (9 GS, 1 ON)	3 (1 GS, 2 ON)	1 (1 GS, 0 ON)	2 (2 GS, 0 ON)	0 (2)	0 (5)
Antiglobulins in peak II	9	1 (1)	1 (2)	0	0 (3)	0 (5)
IgG RRC-Ab in peak I	4	1	0	0	0	0
IgM RRC-Ab in peak II	2	0	0	0	0	0
IgA RRC-Ab in peak I	2 (4)	0 (1)	0 (2)	2	0 (1)	0 (2)

* As judged from the elution prophile only.
§ No. of sera lacking antibodies of the mentioned specificity and/or Ig class are shown in brackets.

an indirect immunofluorescence technique was developed. Rabbit red cells were washed 4 times as described above and 5 μl volumes of pelletted red cells were pipetted into small test tubes. To each tube, 2 drops of fraction were added and, after thorough stirring, the tubes were incubated for 30 minutes at room temperature. After 3 sets of washing in PBS, 2 drops of conjugate diluted 1:10 were added to the pellet and the tubes were again incubated for 30 minutes at room temperature. The cells were washed in PBS 3 times and one drop of glycerol/PBS mixture (1:2) was added to each tube. One drop of cells suspended in this mixture was mounted on slides with a cover slip and the preparations were immediately studied by incident light fluorescence microscopy using a fluorite oil immersion objective, 54 × magnification, numerical aperture 0.95 (Leitz, Wetzlar). Positive reactions were seen as multiple fluorescent patches on the surface of the red cells. The red cell nature of the positive cells was confirmed by phase contrast illumination in transient light Serum from a child with severe combined immunodeficiency and serum from a normal adult person absorbed repeatedly with rabbit red cells served as two negative control sera. Three sera from 2–3 months old children showed weakly positive reactions for IgG RRC-Ab probably derived from the mother and weakly positive reactions for IgM RRC-Ab probably produced by the children themselves (19), but no IgA RRC-Ab.

Very good agreement between the direct haemagglutination results and the indirect immunofluorescence technique for demonstration of IgM RRC-Ab appearing in the fractions of peak I was obtained Titration experiments indicated a comparable sensitivity of the two techniques.

In an effort to elucidate whether the sensitivity of the two indirect immunofluorescence techniques used for demonstration of ANF and RRC-Ab was different, 9 RA whole sera were titrated for IgG ANF and IgG RRC-Ab. The titre ranges were comparable which would indicate that the sensitivity of the techniques is not essentially different.

RESULTS

Upon Sephadex G-200 gel filtration at neutral pH, most sera showed the well-known 3-peaked protein elution prophile characteristic of normal sera (5). Four Felty sera behaved differently in that they showed clearly visible amounts of intermediate size proteins in the region between peak I and II (Fig. 2). In these sera, IgG was demonstrated by double immunodiffusion in all peak I and II frac-

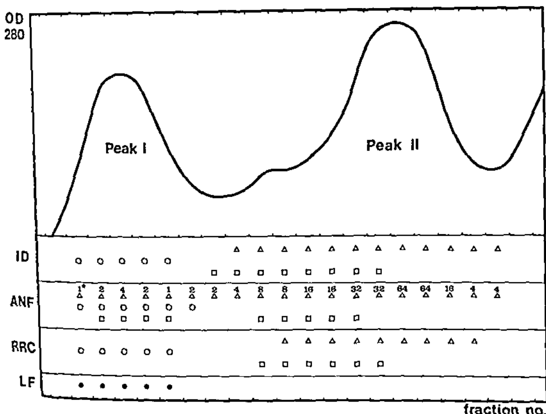


Fig. 1. Serum from a patient with rheumatoid arthritis without neutropenia. 3 ml of serum subjected to Sephadex G-200 gel filtration at pH 7.35 in Tris-HCl buffered saline containing 0 02 M sodium azide/l. ID = double immunodiffusion used to trace the three main Ig classes. ANF = antinuclear factors (in this case strictly granulocyte-specific). RRC = rabbit red cell antibodies. LF = latex fixation test. △ IgG, ○ IgM, □ IgA, ● rheumatoid factor agglutination. * Figures show the IgG granulocyte-specific antinuclear factor titres in each fraction.

tions. Most of the other sera from Felty patients also contained macromolecular and intermediate size IgG as did the sera from patients with neutropenia whether or not chronic arthritis was present (Table 1). Immunochemically, IgM could only be detected in peak I fractions, whereas IgA was found in both peaks. Using the rheumatoid factor precipitation technique, IgG aggregates could be detected in 8 whole sera from Felty patients and 2 sera from RA patients with neutropenia.

In order to elucidate whether the aggregated IgG represented randomly selected IgG molecules with numerous antigenic specificities or selected molecules with restricted specificities, all fractions were studied for presence of IgG ANF and RRC-Ab. IgG ANF were found both in peak I and peak II fractions in 21 out of the 22 rheumatoid sera, indicating participation of these antibodies in the immune complexes (Fig. 1, 2 and Table 1). In 14 sera, the complexed ANF showed granulocyte-specificity. In contrast, macromolecular IgG RRC-Ab were rarely seen (Fig. 2 and Table 1). Titration of IgG GS-ANF and ON-ANF in each of the serum fractions shown in Fig. 1 and 2 disclosed remarkably high titres of IgG GS-ANF in peak I fractions of the Felty serum. The IgG GS-ANF titres were 6 to 7 times higher than the IgG ON-ANF titres in peak I fractions of the Felty serum. The titres of IgG RRC-Ab in this region were very low (Fig. 2).

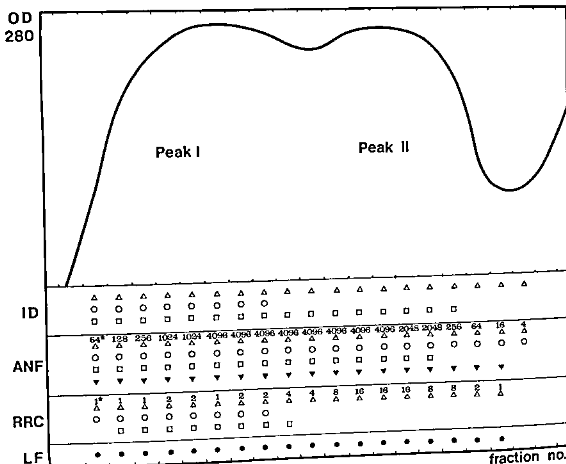


Fig. 2. Serum from a patient with Felty's syndrome. 3 ml of serum fractionated on Sephadex G-200 at pH 7.35. ▼ Complement-fixing granulocyte-specific antinuclear factors. (For other abbreviations and symbols see legend to Fig. 1). * Figures show the IgG granulocyte-specific antinuclear factor titres and the IgG rabbit red cell antibody titres, respectively.

IgM ANF were present in peak I fractions in all patient and control sera, but in addition low molecular size IgM ANF were detected in 18 rheumatoid sera (Fig. 2 and Table 1). In 9 of these sera the IgM ANF were granulocyte-specific. Low molecular size IgM ANF were not seen in any control serum. IgM RRC-Ab were invariably present in peak I fractions of all sera, but only 2 Felty sera contained low molecular size IgM RRC-Ab. Antiglobulins were found in peak I fractions of all sera containing antiglobulins in a titre of ≧32 by the latex fixation slide test. Nine Felty sera and 2 RA sera showed antiglobulin activity in most peak II fractions, indicating low molecular size antiglobulins (Fig. 2 and Table 1). 2 sera showing negative reactions when whole serum was studied revealed presence of antiglobulins in peak I after gel filtration, indicating removal of agglutination inhibitors even at neutral pH. One serum from a Felty patient which repeatedly had shown negative reactions for ANF when whole serum was investigated showed clearly positive reactions for IgG, IgM and IgA GS-ANF after gel filtration at neutral pH, indicating removal of ANF inhibitors from both peaks by gel filtration.

In 18 rheumatoid patients and 2 normal controls, IgA was demonstrated immunochemically in all peak I fractions (Fig. 2 and Table 1). IgA ANF were eluted together with

TABLE 2. *Summary of Physical, Immunochemical and Serological Data Obtained from Gel Filtration Studies of Sera at Acid pH*

	Felty's syndrome	RA with neutropenia	RA without neutropenia	Neutropenia without RA
Number of sera	10	2	2	2
Intermediate size complexes*	0	0	0	0
IgG in peak I	3	1	0	1
IgM in peak II	1	0	0	0
IgA in peak I	4	0	2	1
IgG ANF in peak I	3 (2 GS, 1 ON)	0	1 (1 GS, 0 ON)	0
IgM ANF in peak II	4 (3 GS, 1 ON)	0	1 (1 GS, 0 ON)	0
IgA ANF in peak I	2 (2 GS, 0 ON)	0	0 (1 GS, 0 ON)	0
Antiglobulins in peak II	5 (1)§	0 (1)	0 (1)	0
IgG RRC-Ab in peak I	2	0	0	0
IgM RC-Ab in peak II	0	0	0	0
IgA RRC-Ab in peak I	1 (2)	1	0 (1)	0

* As judged from the elution prophile only.
§ No. of sera lacking antibodies of the mentioned specificity and/or Ig class are shown in brackets.

the macromolecules in 16 of these sera (Fig. 1), IgA RRC-Ab in only 4 of the 15 rheumatoid sera shown to contain such antibodies. GS-ANF were the most common macromolecular IgA ANF to be found in 13 cases.

Since denaturation of IgG in whole serum may lead to irreversible aggregation, 16 of the rheumatoid sera (see Table 2) were subjected to fractionation at acid pH. Disappearance of high molecular size IgG from peak I fractions was interpreted as immune complex dissociation. Three of the 4 Felty sera which contained high amounts of intermediate size IgG at neutral pH were fractionated at acid pH and now showed a normal 3-peaked elution pattern as did all other sera. In most cases, IgG antibodies moved to peak II fractions (Fig. 3, 4 and Table 2). Only 3 Felty sera and one RA serum still contained some macromolecular IgG ANF. Low molecular size IgM ANF disappeared from 9 sera after gel filtration at acid pH. One Felty serum giving strong IgM ANF reactions in most fractions of peak I and II after gel filtration at neutral pH totally lacked IgM ANF activity in all fractions after filtration at acid pH. Complement-fixing GS-ANF behaved as IgG antibodies both at neutral and acid pH fractionation (Fig. 2 and 4). Immunochemically detectable IgA disappeared from the front fractions of peak I in 4 sera, IgA ANF similarly in 4 sera, probably indicating immune complex dissociation.

DISCUSSION

The existence of circulating immune complexes in RA is rather well-substantiated as shown by ultracentrifugation (12, 17), precipitation with isolated IgM rheumatoid factors (8, 26) and C1q (1), platelet aggregation (15), cryoprecipitation (20) and histamine release from guinea pig lung (6, 7). While much attention has been directed to the presence of rheumatoid factors of the 19S and 7S varieties in these complexes, little is known about other specific antibodies which may be regular participants in the complexes.

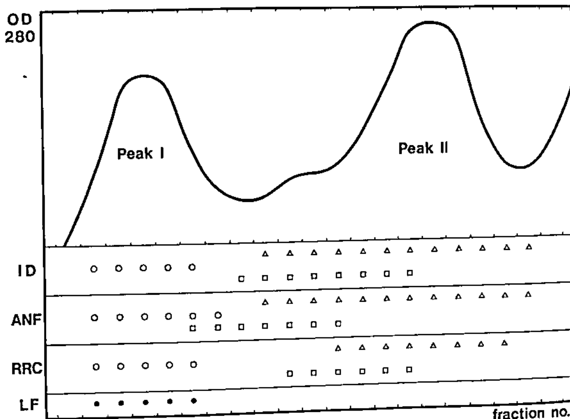


Fig. 3. Serum from the patient with rheumatoid arthritis shown in Fig. 1. 3 ml of serum subjected to fractionation on Sephadex G-200 at pH 4.50 in acetate buffered saline containing 0.02 M sodium azide/l. (For abbreviations and symbols see legend to Fig. 1).

IgG aggregates could be shown in most sera from patients with Felty's syndrome and RA patients with neutropenia by precipitation with monoclonal rheumatoid factor, but the technique was too insensitive to show the IgG aggregates present in virtually all RA sera.

GS-ANF have been shown to be common participants in rheumatoid joint fluid cryoprecipitates (25). These precipitates which may partly represent cold-insoluble immune complexes appeared to be enriched with these antibodies. In patients with Felty's syndrome, cryoglobulins isolated from serum were recently shown to contain high titres of ANF reacting predominantly with granulocyte nuclei (20). The cryoglobulins showed strong complement fixability. We have confirmed this by showing high titres of IgG and IgM GS-ANF in 4 cryoglobulins from sera of Felty patients. The specific antibodies readily fixed complement (21).

Similarly, the gel filtration studies presented here indicate participation of GS-ANF and ON-ANF in the high molecular and intermediate size IgG complexes to be found not only in Felty sera, but in the vast majority of other rheumatoid sera as well. In about 2/3 of the sera the complexed IgG ANF showed granulocyte specificity. Since absorption experiments were not included and titration was not done on peak I fractions of all sera, the prevalence of complexed IgG GS-ANF may be much higher than shown by the study of the undiluted fractions (24). This is illustrated by the finding of ON-ANF in virtually all undiluted peak I fractions of the Felty serum shown in Fig. 2, though titration

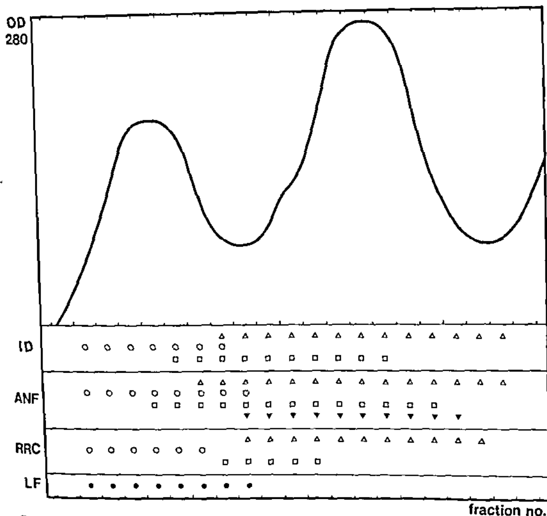


Fig. 4 Serum from the patient with Felty's syndrome shown in Fig. 2. 3 ml of serum subjected to fractionation at pH 4 50 on Sephadex G-200. (For abbreviations and symbols see legend to Fig. 1 and 2).

showed GS-ANF to be present in 6–7 times higher titres.

Theoretical objections may be raised against the legitimacy of comparing titres of a specific antibody in fractions containing complexed and non-complexed antibodies, but it seems permissible to conclude that a fair amount of GS-ANF is found in fractions containing complexed IgG. In contrast, the naturally occurring IgG RRC-Ab as a rule appeared in uncomplexed form or, if complexed, these antibodies were very modestly represented in peak I.

These results indicate a restriction as to the antigenic specificities of the IgG antibodies constituting the complexes. The reason for the restriction is unknown. IgG antibodies which have reacted with a circulating soluble antigen, i.e. nuclear antigen, may have a greater propensity to react with antiglobulins than native IgG antibodies. However, a "core" antigen-antibody complex in a rheumatoid factor containing aggregate has never been shown to exist. It is unlikely that differences in sensitivity of the two immunofluorescence techniques used for the demonstration of ANF and RRC-Ab should account for the antibody restriction found in the IgG com-

plexes since both methods were equally efficient in tracing the two antibodies in the dilution experiments.

7S IgM molecules in sera of some patients with RA have been described, first and foremost those that manifest vasculitis (7, 18). The present study identify some of these monomeric IgM antibodies as ANF. Low molecular size antiglobulins were also found, but it is uncertain whether these belonged to the 7S IgG, IgM or eventually IgA variety. Although IgM ANF were detected in all control sera these antibodies never occurred as 7S molecules.

One can only guess about the reasons why some ANF of the IgM class, contrary to IgM RRC-Ab, assume 7S nature in RA while both of these belonged to the macromolecular form when encountered in normal controls and in patients with osteo-arthrosis. Rheumatoid synovitis is accompanied by a lowering of the pH in the tissue thereby giving rize to a shift in the redox balance towards a more reductive state. The hydrogen excess is associated with an increased leakage of hydrolytic enzymes through the lysosomal membranes into the micro-environment (4). One likely possibility is therefore that 7S IgM ANF result from enzymatic or reductive degradation of complex-bound or locally produced 19S IgM ANF by agents released from inflammatory cells in the course of the inflammatory processes. On the other hand, IgM ANF may be produced locally in the inflamed synovium while IgM RRC-Ab probably is not. The joint fluid content of these antibodies is quite different, the first being present in considerable amounts (24) while the other is barely demonstrable (21), thus supporting the conception of a different origin of these antibodies. If J-chains are not synthesized and/or coupled to the 7S IgM antibodies produced in the synovium, the 7S antibodies in serum may represent a spill-over from the joint.

By gel filtration at neutral pH it was possible to disclose a presence of macromolecular antiglobulins in 2 sera previously shown to give negative reactions at several dilutions of whole serum. This indicates removal of agglutination inhibitors from peak I, probably native IgG (3, 8). Also inhibitors of GS-ANF could be removed both from peak I and peak II by gel filtration at neutral pH, indicating low molecular size of the inhibitors.

IgA was present in the very first fractions of peak I in most rheumatoid sera as were the IgA GS-ANF. In contrast, IgA RRC-Ab were absent from this region in most cases.

Fractionation of 16 rheumatoid sera at acid pH generally resulted in disappearance of macromolecular and intermediate size IgG, suggesting immune complex dissociation (8, 12, 26). IgG ANF were now exclusively found in peak II except in 4 cases which showed persistence of small amounts of non-dissociated IgG probably representing high affinity immune complexes. Also IgA and IgA ANF were displaced to fractions containing somewhat lower molecular size proteins suggesting immune complex dissociation. One Felty serum exhibiting strongly positive reactions for IgM and IgG GS-ANF in most peak I and II fractions upon fractionation at neutral pH showed complete absence of IgM GS-ANF in all fractions after fractionation at acid pH. This points towards dissociation of immune complexes containing IgG GS-ANF as well as IgM antibodies without specificity for nuclear antigens, probably IgM antiglobulins (2, 14). The Ig class nature of a given specific antibody in rheumatoid sera, therefore, can hardly be determined with reasonable certainty if bound in preformed complexes as shown for ANF in this study. Even the coexistence of antibodies to immunoglobulins and/or complement components together with the specific antibody studied may mask the results since these antibodies can couple secondarily to the antigen-antibody complex at the moment of the serological reaction.

7S IgM antibodies disappeared in most cases after gel filtration at acid pH. The 19S form of IgM is quite sensitive to low pH and the present data may reflect an even greater sensitivity of the 7S IgM form.

Complement-fixing GS-ANF shown to be

present in sera from neutropenic cases of RA (23) behaved as if they belonged to the IgG class appearing both in peak I and peak II at neutral pH but only in peak II at acid pH.

The present study indicate a regular involvement of GS-ANF in circulating immune complexes in RA. Hereby the GS-ANF may gain pathophysiological activity by contributing to type III reactions in the vessel walls. In neutropenic cases of RA and related conditions, the antibodies show complement fixability which may augment the phlogogenic effect of the immune complexes.

The helpful advice and criticism of dr. *Viggo Faber* and dr. *Sten Müllertz* is gratefully acknowledged. Dr. *Birger Jensen* is thanked for the gift of isolated IgM from the patient with monoclonal IgM rheumatoid factor in serum. Mrs. *Tove Dietz*, Miss *Berit Madsen* and Mrs. *Merete Skånild* have provided skilful technical assistance for which they are thanked.

This study was supported by the *Danish Research Foundation*, grants no. 512-3212 and 512-3648, and by the *National Association Against Rheumatic Diseases*.

REFERENCES

1. *Agnello, V., Winchester, R. J. & Kunkel, H. G.:* Precipitin reactions of the C1q component of complement with aggregated IgG and immune complexes Immunology *19:* 909–919, 1970.
2. *Barnett, E. V., Condemi, J. J., Leddy, J. P. & Vaughan, J. H.* Gamma$_2$, gamma$_{1A}$ and gamma$_{1M}$ antinuclear factors in human sera. J. Clin. Invest. *43:* 1104–1115, 1964.
3. *Bluestone, R., Goldberg, L. S. & Cracchiolo, A* · Hidden rheumatoid factor in seronegative nodular rheumatoid arthritis. Lancet *ii:* 878–879, 1969.
4. *Chayen, J, Bitensky, L., Butcher, R. G. & Poulter, L. W.*· Redox control of lysosomes in human synovia. Nature (London) *222:* 281–282, 1969.
5. *Fahey, J. L. & Terry, W.:* Ion exchange chromatography and gel filtration. In: *Weir, D M.* (Ed.): Handbook of Experimental Immunology 1st Edition, Blackwell Scientific Publications Ltd., Oxford & Edinburgh 1967, pp. 19–43.
6. *Gordon, D. A., Bell, D. A , Baumal, R. & Broder, I.:* Studies into the occurrence of soluble antigen-antibody complexes in disease. IV. Correlation between the rheumatoid biologically active factor and the clinical features of rheumatoid arthritis. Clin. exp. Immunol. *5:* 57–66, 1969.
7. *Gordon, D. A., Stein, J. L. & Broder, I.:* The extraarticular features of rheumatoid arthritis. A systemic analysis of 127 cases. Amer. J. Med. *54:* 445–452, 1973.
8. *Hannestad, K.:* Rheumatoid factors reacting with autologous native γG-globulin and joint fluid γG aggregates. Clin. exp. Immunol. *3:* 671–690, 1968.
9. *Hart, F. D.:* Rheumatoid arthritis. Extra-articular manifestations. Brit. Med. J. *3:* 131–136, 1969.
10. *Hunder, G. G. & McDuffie, F. C.:* Hypocomplementaemia in rheumatoid arthritis. Amer. J. Med. *54:* 461–472, 1973.
11. *Hurd, E. R., LoSpolluto, J. & Ziff, M.:* The role of immune complexes in the production of the neutropenia of Felty's syndrome. J. Rheumatol. *1:* suppl. 1, 105, 1974 (abstr.).
12. *Kunkel, H. G., Müller-Eberhard, H. J., Fudenberg, H. H. & Tomasi, T. S.:* Gammaglobulin complexes in rheumatoid arthritis and certain other conditions. J. Clin. Invest. *40:* 117–129, 1961.
13. *Laurell, C.-B.:* Antigen-antibody crossed electrophoresis. Analyt. Biochem. *10:* 358–361, 1965.
14. *McCormick, J. N. & Day, J.:* The association of rheumatoid factor with antinuclear factor activity. Lancet *ii:* 554–556, 1963.
15. *Norberg, R.:* IgG complexes in serum of rheumatoid arthritis patients. Scand. J. Immunol. *3:* 229–236, 1974.
16. *Ropes, M. V., Bennett, G. A., Cobb, S., Jacox, R. & Jessar, R. A.:* 1958 revision of the diagnostic criteria for rheumatoid arthritis. Arthritis Rheum. *2:* 16–18, 1958
17. *Schrohenloher, R. E.:* Characterization of the γ-globulin complexes present in certain sera having high titres of anti-γ-globulin activity. J. Clin. Invest. *45:* 501–512, 1966.
18. *Stage, D. E. & Mannik, M.:* 7S γM-globulin in rheumatoid arthritis: Evaluation of its clinical significance. Arthritis Rheum. *14:* 440–450, 1971.
19. *Tønder, O., Natvig, J. B. & Matre, R.:* Antibodies in human sera to rabbit erythrocytes. Immunology *12*. 629–637, 1967.
20. *Weisman, M. H. & Zvaifler, N. J.:* Immunologic studies of Felty's syndrome. J. Rheumatol. *1:* suppl. 1, 105, 1974 (abstr.).
21. *Wiik, A.:* Unpublished work.
22. *Wiik, A. & Munthe, E.:* Restrictions among heavy and light chain determinants of granulocyte-specific antinuclear factors. Immunology *23:* 53–60, 1972.
23. *Wiik, A. & Munthe, E.:* Complement-fixing granulocyte-specific antinuclear factors in neu-

23*

tropenic cases of rheumatoid arthritis. Immunology *26:* 1127–1134, 1974.

24. *Wiik, A., Jensen, E.* & *Friis, J.:* Granulocyte-specific antinuclear factors in synovial fluids and sera from patients with rheumatoid arthritis. Ann. rheum. Dis. *33:* 515–522, 1974.
25. *Wiik, A., Jensen, E., Friis, J.* & *Bach-Andersen, R.:* Participation of granulocyte-specific antinuclear factors in rheumatoid joint fluid cryoprecipitates. Acta path. microbiol. scand. Sect. C, *83:* 265–272, 1975.
26. *Winchester, R. J., Kunkel, H. G.* & *Agnello, V.:* Occurrence of γ-globulin complexes in serum and joint fluids of rheumatoid arthritis patients: Use of monoclonal rheumatoid factors for their demonstration. J. exp. Med. *134:* 286–295, 1971.

Acta path. microbiol. scand. Sect. C, *83:* 365–369, 1975

JOINT FLUID IMMUNE COMPLEXES INVOLVING GRANULOCYTE-SPECIFIC ANTINUCLEAR FACTORS IN RHEUMATOID ARTHRITIS

ALLAN WIIK

Immunological Laboratory, University Clinic for Infectious Diseases and Department of Clinical Chemistry, Blegdamshospitalet, Copenhagen, Denmark

Wiik, A. Joint fluid immune complexes involving granulocyte-specific antinuclear factors in rheumatoid arthritis. Acta path. microbiol. scand. Sect. C, *83:* 365–369, 1975.

Joint fluids from nine patients with rheumatoid arthritis and three patients with osteoarthrosis were fractionated by Sephadex G-200 gel filtration at pH 7.35. Most rheumatoid joint fluids contained macromolecular and intermediate size aggregates of IgG, whereas osteo-arthrosis joint fluids did not. The aggregates regularly comprised IgG granulocyte-specific antinuclear factors, but only rarely any naturally occurring antibodies to rabbit red cells. Inhibitors of granulocyte-specific antinuclear factor reactions were removed from one rheumatoid joint fluid by gel filtration at neutral pH. IgM antinuclear factors of low molecular size were detected in five rheumatoid joint fluids. Three aggregates containing rheumatoid joint fluids were also fractionated at pH 4.5. This caused dissociation of the aggregates in one case and incomplete dissociation in the two other. The low molecular size IgM antinuclear factors disappeared, probably indicating pronounced sensitivity of these antibodies to lowering of the pH. The data suggest participation of granulocyte-specific antinuclear factors in rheumatoid joint fluid immune complexes. These antibodies may thus gain pathophysiological activity by contributing to immune complex mediated inflammation.

Key words: Rheumatoid arthritis; granulocyte-specific antinuclear factors; immune complexes; joint fluid.

A. Wiik, Immunological Laboratory, University Clinic for Infectious Diseases, Blegdamshospitalet, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark.

Received 18.iv 75 Accepted 24.v.75

The aim of this study is to confirm and extend previous observation suggesting participation of granulocyte-specific and organnonspecific antinuclear factors (GS-ANF and ON-ANF) in immune complexes of rheumatoid synovial fluid (SF) in an effort to support the conception that these antibodies may be essential contributors to immune complex induced inflammatory processes in rheumatoid arthritis (RA) (2, 9, 10, 13).

MATERIALS AND METHODS

SF was obtained from nine patients who satisfied the criteria for classical or definite RA (5), and from three patients with osteo-arthrosis featuring typical clinical and radiological signs of this disease. The SF specimens were stabilized with EDTA

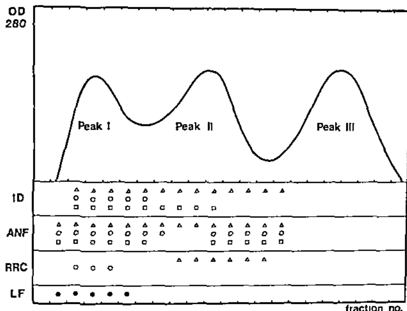


Fig. 1. Joint fluid from a rheumatoid arthritis patient subjected to Sephadex G-200 gel filtration at pH 7.35 in Tris-HCl buffered saline containing 0.01 M EDTA/l and 0.02 M sodium azide/l. ID = double immunodiffusion used to trace the three main Ig classes. ANF = antinuclear factors. RRC = rabbit red cell antibodies. LF = F II latex fixation test. △ IgG, ○ IgM, □ IgA, ● rheumatoid factor agglutination.

0.01 mM/ml, were cleared from cells by centrifugation and were treated with testicular hyaluronidase as described earlier (10). All samples were stored for a period not exceeding a few months until fractionation could be carried out.

3 ml of each SF was fractionated by gel filtration on Sephadex G-200 at pH 7.35 using a Tris-HCl elution buffer containing 0.01 M EDTA and 0.02 M sodium azide/l, $I = 0.154$ as previously described (8). Three RA SF which were found to contain aggregates of IgG at neutral pH were also fractionated at pH 4.5 using an acetate buffer containing 0.01 M EDTA and 0.02 M sodium azide/l, $I = 0.154$ (8). All fractions were neutralized with NaOH shortly after completion of the fractionation, the final pH being 7.3 ± 0.2.

All fractions were studied for presence of immunoglobulins, GS-ANF, ON-ANF, antiglobulins and heterophilic antibodies to rabbit red cells (RRC-Ab) using methods described in detail elsewhere (8, 9, 11, 12).

RESULTS

The protein elution pattern was in all cases very similar to that of normal serum fractionated on G-200 Sephadex (Fig. 1), the proteins emerging as three main peaks. Double immunodiffusion assays showed IgG and IgA in both peak I and peak II fractions, while IgM was only revealed in peak I fractions by this method (Fig. 1 and Table 1).

In all RA SF which in precedent studies had been shown to contain IgG ANF such antibodies were present not only in peak II, as could be expected, but also in peak I and in the region between peaks I and II, suggesting that some IgG ANF in aggregated form existed as high molecular and intermediate size aggregates. Similar results were obtained as regards IgA ANF which could be traced in all the fractions of peak I in the six SF containing this antibody. In most cases, the IgG and IgA ANF showed granulocyte specificity.

Macromolecular IgM ANF were found in all of the RA SF, but in addition, five SF contained low molecular size IgM ANF (Fig. 1 and Table 1).

One RA SF exhibited no ANF activity at all when the unfractionated specimen was studied. However, after gel filtration at neutral pH, this SF showed presence of both IgG, IgM and IgA GS-ANF, indicating a removal of inhibitors from both peaks.

TABLE 1. *Summary of Essential Physical, Immunochemical and Serological Data Obtained from Gel Filtration Studies of Synovial Fluids at Neutral and Acid pH*

	Fractionation at neutral pH		Fractionation at acid pH
	Rheum. arthr.	Osteoarthr.	Rheum. arthr.
Number of sera	9	3	3
IgG in peak I	9	0	2
IgM in peak II	0	0	0
IgA in peak I	8	0	2
IgG ANF in peak I	7 5 GS 2 ON (2)*	0 (3)	2 2 GS 1 ON
IgM ANF in peak II	5 2 GS 3 ON	0 (3)	0 (1)
IgA ANF in peak I	6 6 GS 0 ON (3)	0 (3)	0 (2)
Antiglobulins in peak II	0 (1)	0 (3)	1 (1)
IgG RRC-Ab in peak I	1 (1)	0	0
IgM RRC-Ab in peak II	0	0	0
IgA RRC-Ab in peak I	0 (8)	0 (3)	0 (3)

* No. of sera lacking antibodies of the mentioned specificity and/or Ig class are shown in brackets.

Antiglobulins could be demonstrated in all RA SF, but none of the OA SF. These antibodies were in all cases restricted to peak I fractions at neutral pH.

Though IgG RRC-Ab could be detected in all OA and 8 RA SF, these antibodies usually appeared in peak II fractions only, indicating that the aggregates did not comprise antibodies of this specificity (Fig. 1 and Table 1). IgM RRC-Ab could be detected in all OA and RA SF, though mostly the reactions were very weak. IgM RRC-Ab always were of macromolecular nature only.

Fractionation of RA SF at pH 4.5 caused displacement of all IgG and IgA ANF activity to peak II fractions in one case, while two cases showed persistence of some IgG GS-ANF in peak I (Fig. 2 and Table 1). One RA SF, which exhibited strongly positive reactions of IgG, IgA and IgM GS-ANF upon fractionation at neutral pH (Fig. 1), was found to have only IgG GS-ANF activity left when studied after fractionation at acid pH (Fig. 2). 7S IgM ANF, present in two of the SF at neutral pH, disappeared after gel filtration at acid pH.

DISCUSSION

It is generally assumed that immune complexes in the synovial membrane, the SF and the circulation may play a role in the initiation and perpetuation of rheumatoid inflammation (reviewed in 4, 13). Antiglobulins of IgG and IgM class nature are known to be regular participants in the immune complexes, but only little information is available about other specific antibodies which may be essential elements in the complexes.

Previous studies have suggested that GS-ANF may be common constituents of circulating immune complexes in RA and Felty's syndrome (7, 8). GS-ANF are also frequently encountered in rheumatoid SF cryoglobulins which in part may represent cold-insoluble immune complexes (10). The present data appear to support the hypothesis that GS-ANF and ON-ANF through participation in immune complexes of RA SF may contribute to the inflammatory reactions in the synovial environment (10). The discovery that a normally occurring heterophilic antibody such as IgG RRC-Ab mostly does not take part in

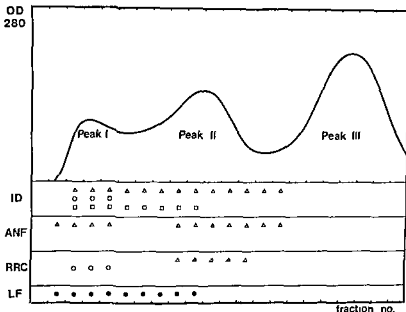


Fig. 2. Joint fluid from the rheumatoid arthritis patient shown in Fig. 1 subjected to fractionation on Sephadex G-200 at pH 4.50 in acetate buffered saline containing 0.01 M EDTA/l and 0.02 M sodium azide/l. (For abbreviations and symbols see legend to Fig. 1).

the aggregates points towards restriction as to the antigenic specificities of the antibodies comprised in the immune complexes (8).

The Sephadex G-200 gel filtration technique has previously been shown to be valuable for the removal of ANF inhibitors from serum (8) and this method proved valuable for unmasking the presence of GS-ANF in SF as well. The GS-ANF inhibitors most probably interact with GS-ANF through a very loose binding since it was possible to remove the inhibitory activity at a physiological pH under isotonic conditions. However, it cannot be excluded that the inhibitors exert their activity through a competitive blocking of the nuclear antigens in the cell substrate.

The dissociation experiments may illustrate the notion that antiglobulins, participating in the complexe[illegible] can mask the real Ig class nature of a spe[illegible]fic antibody (1, 3) since IgM and IgA anti[illegible]es without ANF activity of their own wer[illegible]moved by gel filtration at acid pH, leavin[illegible] GS-ANF behind as the only GS-ANF p[illegible] At the same time, antiglobulin activit[illegible]s extended to include fractions containing intermediate size proteins (Fig. 2).

The immune complexes encountered in RA sera readily dissociate at pH 4.5 (8). The present data indicate that the IgG aggregates of RA SF are not quite easily dissociated which may either be taken to indicate a higher affinity of the antibodies forming the complexes or a certain degree of irreversible aggregation due to denaturation. However, aggregation was not seen in any OA SF, suggesting that inadequate handling of the specimens could not account for the aggregation.

Low molecular size IgM ANF have previously been found in systemic lupus erythematosus and RA sera (6, 8) and the present investigation shows that they are common in RA SF as well. Their derivation and clinical significance are unknown. They disappeared after gel filtration at acid pH, probably indicating pronounced sensitivity to lowering of pH.

In conclusion, the present data confirm and extend earlier observations of the participation of GS-ANF in immune complexes

of RA patients suggesting that these antibodies may have pathogenetic significance by contributing to immune complex mediated inflammatory reactions.

Dr. *Viggo Faber* is thanked for carefully reviewing the manuscript, dr. *Erik Jensen* for performing the arthrocenteses. The skilful technical assistance of Mrs. *Ulla Høiby*, Miss *Berit Madsen* and Mrs. *Tove Dietz* is gratefully acknowledged.

This study was supported by the *National Danish Association Against Rheumatic Diseases* and the *Danish Medical Research Council*, grant no. 512-3212 and 512-3648.

REFERENCES

1. *Barnett, E. V., Condemi, J. J., Leddy, J. P. & Vaughan, J. H.:* Gamma$_{2}$, gamma$_{1A}$ and gamma$_{1M}$ antinuclear factors in human sera. J. Clin. Invest. *43:* 1104–1115, 1964.
2. *Cracchiolo, A., Goldberg, L. S., Barnett, E. V. & Bluestone, R.:* Studies of cryoprecipitates from synovial fluid of rheumatoid patients. Immunology *20:* 1067–1077, 1971.
3. *McCormick, J. N. & Day, J.:* The association of rheumatoid factor with antinuclear factor activity. Lancet ii: 554–556, 1963.
4. *Munthe, E.:* Immunological Studies in Rheumatoid Arthritis with Special Reference to Autoantibodies and Immune Complexes in the Tissue. Universitetsforlaget, Oslo, 1972. Thesis.
5. *Ropes, M. V., Bennett, G. A., Cobb, S., Jacox, R. & Jessar, R. A.:* 1958 revision of the diagnostic criteria for rheumatoid arthritis. Arthritis Rheum. *2:* 16–18, 1958.
6. *Rothfield, N. F., Frangione, B. & Franklin, E. C.:* Slowly sedimenting mercaptoethanol resistant antinuclear factors related antigenically to M immunoglobulins (γ_{1M}-globulin) in patients with systemic lupus erythematosus. J. Clin. Invest. *44:* 62–72, 1965.
7. *Weisman, M. H. & Zvaifler, N. J.:* Immunologic studies of Felty's syndrome. J. Rheumatol. *1:* suppl. 1, 105, 1974 (abstr.).
8. *Wiik, A.:* Circulating immune complexes involving granulocyte-specific antinuclear factors in Felty's syndrome and rheumatoid arthritis. Acta path. microbiol. scand. Sect. C, *83:* 354–364, 1975.
9. *Wiik, A., Jensen, E. & Friis, J.:* Granulocyte-specific antinuclear factors in synovial fluids and sera from patients with rheumatoid arthritis. Ann. rheum. Dis. *33:* 515–522, 1974.
10. *Wiik, A., Jensen, E., Friis, J. & Bach-Andersen, R.:* Participation of granulocyte-specific antinuclear factors in rheumatoid joint fluid cryoprecipitates. Acta path. microbiol. scand. Sect. C, 83: 265–272, 1975.
11. *Wiik, A. & Munthe, E.:* Restrictions among heavy and light chain determinants of granulocyte-specific antinuclear factors. Immunology *23:* 53–60, 1972.
12. *Wiik, A. & Munthe, E.:* Complement-fixing granulocyte-specific antinuclear factors in neutropenic cases of rheumatoid arthritis. Immunology *26:* 1127–1134, 1974.
13. *Zvaifler, N. J.:* The immunopathology of joint inflammation in rheumatoid arthritis. Advan. Immunol. *16:* 265–336, 1973.

Acta path. microbiol. scand. Sect. C, *83:* 370–376, 1975

THE MECHANISM OF THE L-AGGLUTINATION OF STREPTOCOCCI BY SERA FROM PATIENTS WITH RHEUMATOID ARTHRITIS

POUL CHRISTENSEN*, VIVI-ANNE OXELIUS* and MIMI HOIER-MADSEN**

* The Department of Medical Microbiology, University of Lund, Sweden and
** the Blood Bank & Blood Grouping Department, the State Serum Institute, Copenhagen, Denmark

Christensen, P., Oxelius, V.-A. & Høier-Madsen, M. The mechanism of the L-agglutination of streptococci by sera from patients with rheumatoid arthritis. Acta path. microbiol. scand. Sect. C, *83:* 370–376, 1975.

The L-agglutination of streptococci group A, strain S.F. 130, type T 1 by sera from patients with rheumatoid arthritis was investigated. The IgG fraction of these sera alone could agglutinate the streptococci. Absorption of the sera with the streptococci turned the L-agglutination test negative. On addition of pooled human IgG the test was again positive while IgG myeloma protein had no such effect. These findings indicated participation of IgG antibodies to streptococcal antigens and that these antibodies were present also in serum from healthy individuals. If IgG of subclass 1, 2 and 4 was eliminated from the rheumatoid arthritis sera by absorption with Cowan I staphylococci, the L-agglutination could not be restored by addition of pooled human IgG. Together with findings of other authors, this result indicated participation of anti-IgG of IgG class in the L-agglutination. Furthermore, removal of the broth in which the streptococci were cultured also turned the L-agglutination test with rheumatoid arthritis sera negative, but on replacement with sterile filtered supernatant from gramnegative rods the test again turned positive. The interaction of these factors in the L agglutination—i.e. normally occurring antibodies to streptococcal antigens, anti-IgG of IgG class present in rheumatoid arthritis sera and soluble antigens in the culture supernatant—is discussed

Key words: Streptococci; agglutinability, immune complexes; rheumatoid arthritis.

Poul Christensen, Department of Medical Microbiology, Sölvegatan 23, S-223 62 Lund, Sweden.

eceived 2.v.75 Accepted 10.vi.75

Agglutination of a streptococcal strain by :ra from patients with rheumatoid arthritis 'as first reported in 1931 by *Cecil et al.* It was soon realized that such sera agglutinated a wide variety of streptococci (*Dawson et al.* 1932 a, b, 1934, *Dawson & Olmstead* 1936), while agglutination by sera from patients with streptococcal infections but without rheumatoid arthritis was relatively rare. The streptococcal strain most often used has been group A, type T 1.

The agglutination of living streptococci (the L-agglutination) was long ascribed to specific antibodies to the streptococci (for review, see *Thulin* 1948). Later the agglutina-

tion was thought to be mediated by a reaction between specific antibodies attached to the streptococci and "rheumatoid factors" in the patients' sera; the amount of specific antibodies to the streptococci in sera from patients with rheumatoid arthritis did not exceed that in normal sera (*Lamont-Havers* 1955). *Otto et al.* (1971) stated that both this type of reaction and a direct effect of streptococcal antibodies are involved in the agglutination. On the other side, *Seidel & Knöll* (1972) maintained that: "Type specific T-antibodies are responsible for the L-agglutination with streptococcus pyogenes type 1. The rheumatoid factors are not involved in the L-agglutination".

In view of these conflicting reports it was thought legitimate to investigate the mechanism of the agglutination, especially in the light of the new reports of the interaction between streptococci and immunoglobulins, in which the antibody combining sites are not involved (*Kronvall* 1973, *Christensen & Kronvall* 1974, *Christensen & Oxelius* 1974 and 1975, *Christensen* 1975). In the present paper, the reaction standardized by *Kalbak* (1948) was used.

MATERIALS AND METHODS

L-Agglutination Test ad modum Kalbak

The L-agglutination test with streptococcus group A, type T 1, strain S.F. 130 (Copenhagen), originally belonging to *Griffith's* type strains (*Griffith* 1926), was performed as described by *Kalbak* (1948). The standard suspension of streptococci consisted of living bacteria suspended in the Orthana broth in which they were cultured (*Kalbak* 1948).

Strains

Besides the Copenhagen S.F. 130 strain, another S.F. 130 strain was kindly supplied from the Colindale Reference Laboratory, London. Our extracts prepared as described by *Lancefield* (1928), were investigated by *Dr. W. R. Maxted*, Colindale, for precipitation by anti-M type 1, 3, 6 and 12 serum, the S.F. 130 (Colindale) strain contained M 1 protein (hereinafter called S.F. 130, M+ve), while the Copenhagen strain did not (termed S.F. 130, M−ve). *Dr Maxted* also kindly performed a test for M-associated protein, which was found only in the M+ve strain extract.

The group B, C, D and G strains and the other group A streptococci used in the experiments were isolated from routine bacteriological specimens; they were grouped as described previously (*Christensen et al.* 1973). These strains were tested for uptake of IgG myeloma protein directly after isolation. An *E. coli* and a *Proteus mirabilis* strains were used in some experiments; they were grown on tryptone media (Bacto).

Human Sera and Immunoglobulin Preparations

IgG myeloma proteins were purified and subclass-typed as described earlier (*Christensen & Oxelius* 1974). Gm typing was performed according to *Grubb & Laurell* (1956); two myeloma proteins of allotypes Gm (1) and Gm (4), respectively, of IgG subclass 1 and one IgG myeloma protein of each subclass 2, 3 and 4 were used. The Gm (4) myeloma protein was labelled with ^{125}I (*McConahey & Dixon* 1966).

Commercial, pooled human IgG was purchased from AB Kabi, Stockholm, Sweden (batch No. 44791).

Sera from patients with rheumatoid arthritis were randomly selected; the sera showed titers higher than 1:20 in the L-agglutination test and higher than 1:32 in the *Waaler-Rose* test.

Fractionation of Sera on Sephadex G-200 Column

Chromatography was performed on a Sephadex G-200 column (Pharmacia, Uppsala, Sweden) as described by *Flodin & Killander* (1962). The fractions in the first half of the first peak (corresponding to 19S) were concentrated to the original serum volume on an ultrafiltration cell (Diaflo XM 50) and termed peak I, and all the fractions in the second peak (corresponding to 7S) were pooled and concentrated.

The presence of IgG or IgM in the peaks was demonstrated by immunodiffusion with specific rabbit anti-human IgG and IgM sera, respectively (*Ouchterlony* 1949).

Quantitation of Human IgG

The IgG was quantitated by the electroimmuno assay (*Laurell* 1965).

Absorption Experiments with Streptococci or Staphylococci

Absorption of sera with Cowan I staphylococci to remove IgG subclass 1, 2 and 4 was performed as described earlier (*Ankerst et al.* 1974).

Absorption with S.F. 130, M−ve streptococci was performing according to *Müller* (1962).

Acta path. microbiol. scand. Sect. C, *83:* 370–376, 1975

THE MECHANISM OF THE L-AGGLUTINATION OF STREPTOCOCCI BY SERA FROM PATIENTS WITH RHEUMATOID ARTHRITIS

Poul Christensen*, Vivi-Anne Oxelius* and Mimi Hoier-Madsen**

* The Department of Medical Microbiology, University of Lund, Sweden and
** the Blood Bank & Blood Grouping Department, the State Serum Institute, Copenhagen, Denmark

Christensen, P., Oxelius, V.-A. & Høier-Madsen, M. The mechanism of the L-agglutination of streptococci by sera from patients with rheumatoid arthritis. Acta path. microbiol. scand. Sect. C, *83: 370–376, 1975.*

The L-agglutination of streptococci group A, strain S.F. 130, type T 1 by sera from patients with rheumatoid arthritis was investigated. The IgG fraction of these sera alone could agglutinate the streptococci. Absorption of the sera with the streptococci turned the L-agglutination test negative. On addition of pooled human IgG the test was again positive while IgG myeloma protein had no such effect. These findings indicated participation of IgG antibodies to streptococcal antigens and that these antibodies were present also in serum from healthy individuals. If IgG of subclass 1, 2 and 4 was eliminated from the rheumatoid arthritis sera by absorption with Cowan I staphylococci, the L-agglutination could not be restored by addition of pooled human IgG. Together with findings of other authors, this result indicated participation of anti-IgG of IgG class in the L-agglutination. Furthermore, removal of the broth in which the streptococci were cultured also turned the L-agglutination test with rheumatoid arthritis sera negative, but on replacement with sterile filtered supernatant from gramnegative rods the test again turned positive. The interaction of these factors in the L-agglutination—i.e. normally occurring antibodies to streptococcal antigens, anti-IgG of IgG class present in rheumatoid arthritis sera and soluble antigens in the culture supernatant—is discussed.

Key words: Streptococci; agglutinability; immune complexes; rheumatoid arthritis.

Poul Christensen, Department of Medical Microbiology, Sölvegatan 23, S-223 62 Lund, Sweden.

Received 2 v.75 Accepted 10.vi.75

Agglutination of a streptococcal strain by sera from patients with rheumatoid arthritis was first reported in 1931 by *Cecil et al.* It was soon realized that such sera agglutinated a wide variety of streptococci (*Dawson et al.* 1932 a, b, 1934, *Dawson & Olmstead* 1936), while agglutination by sera from patients with streptococcal infections but without rheumatoid arthritis was relatively rare. The streptococcal strain most often used has been group A, type T 1.

The agglutination of living streptococci (the L-agglutination) was long ascribed to specific antibodies to the streptococci (for review, see *Thulin* 1948). Later the agglutina-

tination test turned negative; addition of an IgG myeloma protein, Gm (4) did not restore the L-agglutination. Similar results were obtained with Gm (1) myeloma protein and rheumatoid arthritis serum containing anti Gm (1).

The Capacity of some Streptococci to Take up ^{125}I Labelled IgG Myeloma Protein

The uptake of ^{125}I labelled IgG myeloma protein by 13 group A streptococci, 14 B, 3 C, 5 D and 3 G, freshly isolated from routine bacteriological specimens is shown in Fig. 1. A marked difference was found between A, C and G streptococci, on one hand, and B and D, on the other. The uptake of IgG by S.F. 130, M−ve was low, that by M+ve was considerable (Fig. 1).

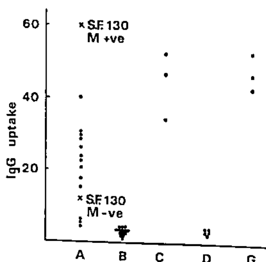


Fig. 1. The uptake of ^{125}I labelled IgG myeloma protein by some streptococci freshly isolated from routine bacteriological specimens. The uptake is given in per cent of 1 μ ^{125}I labelled IgG added.

Substitution of the S.F. 130, M−ve Supernatant with Supernatant from Cultures of Gramnegative Rods in the L-Agglutination

The streptococci from the standard suspension were separated from the Orthana broth in which they had been grown, washed 3 times in 0.9 per cent NaCl and suspended in fresh Orthana broth; no L-agglutination was seen after this treatment. Replacement of the streptococcal supernatant removed by an equal volume of sterile filtered supernatant from an over-night culture of *E. coli* and *Proteus mirabilis* restored the titer of the L-agglutination by 10 sera from patients with rheumatoid arthritis; control with freshly prepared tryptone media was negative. Thus, the sera, absorbed with S.F. 130, M−ve streptococci, turned positive in the L-agglutination test when the supernatant from gramnegative strains was added to the washed S.F. 130, M ve streptococci.

DISCUSSION

I Serum Factors Participating in L-Agglutination

It has been shown (*Thulin* 1955) that the immunoglobulins responsible for the L-agglutination and the agglutination of sensitized red cells belong to different electrophoretic fractions of serum. This finding was corroborated by experiments with rheumatoid arthritis sera with 2-mercaptoethanol (*Otto et al.* 1971, *Seidel* & *Knöll* 1972, *Knöll* & *Tanner* 1968), from which it was postulated that the "rheumatoid factors" belonging to the IgM class are not involved in L-agglutination. Our results showed that the IgG fraction alone can induce L-agglutination, while only fractions containing IgM can agglutinate sensitized sheep red cells.

By absorption of rheumatoid arthritis sera with S.F. 130, M−ve streptococci, the positive L-agglutination turned negative, in accordance with the findings of *Lamont-Havers* (1955). *Lamont-Havers* showed that serum from healthy individuals can restore the L-agglutination of rheumatoid arthritis sera after such absorption. We found that only a minor fraction of IgG was removed by absorption of the sera with S.F. 130, M−ve and that pooled human IgG could restore the L-agglutination in contrast to IgG myeloma proteins. These findings indicated that IgG antibodies to streptococcal antigens participated in the L-agglutination performed as described by *Kalbak* (1948); a larger amount

of IgG would have been removed by absorption with the M+ve variant as the M+ve variant had higher capacity to bind IgG without participation of the antibody combining sites (see *Christensen & Oxelius* 1974). The results of *Lamont-Havers* (1955) as well as those of ours indicated that the IgG antibodies specific to streptococcal antigens were present in the sera from healthy individuals; it is well known, that the sera of healthy human beings contain antibodies to streptococcal antigens, especially extracellular antigens (see *Halbert* 1964, *Rotta* 1972).

After absorption of IgG of subclasses 1, 2 and 4 from the sera with protein A containing staphylococci, pooled human IgG did not restore the L-agglutinating capacity of the sera. Thus, L-agglutination required the presence not only of what we suggested as antibodies to streptococcal antigens but also another IgG factor. To our opinion this factor was anti-IgG of IgG class: in the L-agglutination test, performed as described by *Kalbak* (1948), this factor was not removed by absorption with streptococcal antigens and required the presence of anti-streptococcal antibodies of IgG class to express itself in the L-agglutination. Furthermore, *Lamont-Havers* (1955) showed, that the L-agglutination disappeared after absorption of the rheumatoid arthritis sera with heat-aggregated "gammaglobulin". The presence of anti-IgG of IgG class in the sera from patients with rheumatoid arthritis has *i.a.* been demonstrated by *Torrigiani & Roitt* (1967).

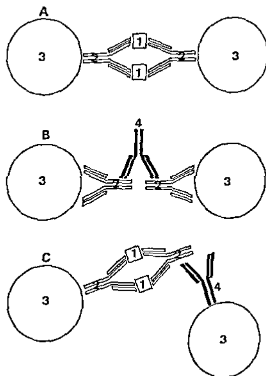


Fig. 2. Theoretically possible reactions participating *in the L-agglutination performed as described by Kalbak* (1948). 1. Streptococcal extracellular antigens. 2. IgG antibodies to streptococcal antigens. 3. streptococci. 4. anti-IgG of IgG class.

2. *Investigation of Streptococcal Strains*

Removal of the supernatant from the S.F. 130, M−ve standard suspension turned the L-agglutination test negative in experiments with rheumatoid arthritis sera. *Thulin* (1948) felt, that this was due to an antigen (the L-antigen) localized to the surface of the streptococci and that this antigen could be removed by washing and centrifugation. We found, however, that the supernatant from cultures of gramnegative rods could restore the L-agglutination. A possible explanation is that immune complexes were formed between soluble antigens and antibodies to these antigens. Recently, some streptococci were shown to be agglutinated by immune complexes (*Christensen* 1975); agglutination of the streptococci by immune complexes was dependent on, among other things, the ability of the streptococci to bind IgG without involving the antibody combining sites (*Christensen* 1975). We found that group A, C and G streptococci bound more ^{125}I labelled IgG myeloma protein than group B and D streptococci. It is well known that A, C and G streptococci are more suitable than B and D streptococci for the L-agglutination test (*Kalbak* 1948).

The S.F. 130, M−ve strain used, combined with ^{125}I labelled IgG myeloma proteins much less readily than the M+ve strain. It is not clear why this strain had lower capacity

to bind ^{125}I labelled IgG myeloma protein than the M+ve strain. The strain is, however, capable to agglutinate sensitized sheep red cells (*Christensen*, unpublished observation). It was not possible to compare the M+ve and M−ve strains regarding L-agglutination as the M+ve strain showed spontaneous agglutination.

The different reactions, possibly participating in the L-agglutination are shown in Fig. 2. The experiments showed that both the supernatant from the streptococcal culture and the fractions of IgG referred to as streptococcal antibodies as well as anti IgG are necessary components in the L-agglutination. Hence, L-agglutination might be caused by the mechanisms illustrated in Fig. 2 A and 2 B together, or by those given in Fig. 2 C; in the model proposed in Fig. 2 B, anti IgG of IgM class might also participate.

This investigation was supported by a grant from "*Riksföreningen mot rheumatism*", Sweden.

REFERENCES

Ankerst, J., Christensen, P., Kjellén, L. & Kronvall, G.: A routine diagnostic test for IgA and IgM antibodies to rubella virus: absorption of IgG with *Staphylococcus aureus*. J. Infec. Dis *130:* 268–273, 1974.

Cecil, R. L., Nicholls, E. E. & Stainsby, W. J.: The etiology of rheumatoid arthritis. Am. Journ. Med. Scien. *181:* 12–25, 1931.

Christensen, P.: Agglutinability of some selected streptococci by immune complexes. Acta path. microbiol. scand. Sect. C, *83:* 28–34, 1975.

Christensen, P., Kahlmeter, G., Jonsson, S. & Kronvall, G.: New method for the serological grouping of streptococci with specific antibodies adsorbed to protein A-containing staphylococci. Inf. Immun *7:* 881–885, 1973.

Christensen, P. & Kronvall, G.: Capacity of some group A, B, C, D and G streptococci to agglutinate sensitized sheep red cells. Acta path. microbiol scand. Sect. B, *82:* 19–24, 1974.

Christensen, P. & Oxelius, V.-A.: Quantitation of the uptake of human IgG by some streptococci groups A, B, C and G. Acta path. microbiol. scand Sect. B, *82:* 475–483, 1974.

Christensen, P. & Oxelius, V.-A.: A reaction between some streptococci and IgA myeloma proteins. Acta path. microbiol. scand. Sect. C, *83:* 184–188, 1975.

Dawson, M. H. & Olmstead, M.: Agglutination and precipitation reactions in rheumatoid arthritis with hemolytic streptococci groups A to G. Proc. Soc. Exper. Biol. Med. *34:* 80–82, 1936.

Dawson, M. H., Olmstead, M. & Boots, R. H.: Agglutination reactions in rheumatoid arthritis; I. Agglutination reactions with streptococcus hemolyticus. J. Immunol. *23:* 187–204, 1932 a.

Dawson, M. H., Olmstead, M. & Boots, R. H.: Agglutination reactions in rheumatoid arthritis; II. The nature and significance of agglutination reactions with streptococcus hemolyticus. J. Immunol. *23:* 205–228, 1932 b.

Dawson, M. H., Olmstead, M. & Boots, R. H.: Agglutination reactions in rheumatoid arthritis; III. Comparison of agglutinins and precipitins for streptococcus hemolyticus in rheumatoid arthritis sera. J. Immunol. *27:* 355–365, 1934.

Flodin, P. & Killander, J.: Fractionation of human serum proteins by gel filtration. Biochem. biophys. Acta (Amst.) *63:* 403–410, 1962.

Griffith, F.: Types of haemolytic streptococci in relation to scarlet fever. J. Hyg. *25:* 385–397, 1926.

Grubb, R. & Laurell, A. B.: Heriditary serological human serum groups. Acta path. microbiol. scand. *39:* 390–398, 1956.

Halbert, S. P.: Analysis of human streptococcal infections by immunodiffusion studies of the antibody response. In: Uhr, J. W. (Ed.): The streptococcus, rheumatic fever and glomerulonephritis. The Williams & Wilkins Company. Baltimore 1964, p. 83–165.

Kalbak, K.: Agglutination of hemolytic streptococci (group A) in serum from patients with rheumatoid arthritis. Acta med. scand. *130:* 358–370, 1948.

Knöll, P. & Tanner, E.: Serologische Studie über typenspezifische Antikörper gegen T-Proteine der hämolysierenden A-Streptokokken bei Polyarthritis chronica progressiva und degenerativen Gelenk- und Wirbelsäulenerkrankungen. Z. Immun. Forsch. *136:* 22–31, 1968.

Kronvall, G.: A surface component in group A, C and G streptococci with non-immune reactivity for immunoglobulin G. J. Immunol. *111:* 1401–1406, 1973.

Lamont-Havers, R. W.: Nature of serum factors causing agglutination of sensitized sheep cells and group A hemolytic streptococci. Proc. Soc. exp. Biol. (N.Y.) *88:* 35–38, 1955.

Lancefield, R. C.: The antigenic complex of streptococcus hemolyticus. I. Demonstration of a type-specific substance in extracts of streptococcus hemolyticus. J. Exp. Med. *47:* 91–103, 1928.

Laurell, C.-B.: Antigen-antibody crossed electrophoresis. Anal. Biochem. *10:* 358–361, 1965.

McConahey, P. J. & Dixon, F. J.: A method of

trace iodination of proteins for immunologic studies. *Int. Arch. Allergy 29:* *185–189, 1966.*

Müller, W.: Die Serologie der chronischen Polyarthritis. Springer-Verlag. Berlin-Göttingen-Heidelberg 1962, p. 85–96.

Otto, R., Reske, W., Venner, M. & Schröder, D.: Der Simultannachweis von Rheumafaktor und Streptokokken-Antikörpern in Serum durch trypsin-behandelte Streptokokken. Z. Immun. Forsch. *142:* 230–247, 1971.

Ouchterlony, Ö.: Antigen-antibody reactions in gels. Acta path. microbiol. scand. *26:* 507–524, 1949.

Rotta, J.: Antistreptolysin O surveys in the populations of some Asian and African countries. In: Haverkorn, M. J. (Ed.): Streptococcal disease and the community. Excerpta Medica. Amsterdam. American Elsevier Publishing Co., Inc., New York 1972, p. 145–163.

Seidel, K. & Knöll, P.: Die Bedeutung der Streptokokken-L-Agglutination und ihre Stellung in der serologischen Diagnostik der progressiven *chronischen Polyarthritis.* A. Rheumaforschg. *31:* 36–41, 1972.

Thulin, K. E.: *Serological aspects of hemolytic* streptococci with special reference to the occur*rence of* O, K *and* L *antigens, and some clini*cal applications. Gleerupska Universitetsbokhandeln, Lund, Sweden 1948.

Thulin, K. E.: The reactions of streptococcal agglutination relative to the RAS factor in rheumatoid arthritis. Acta rheum. scand. *1:* 1–21, 1955.

Torrigiani, G. & Roitt, I. M.: Antiglobulin factors in sera from patients with rheumatoid arthritis and normal subjects. Ann. Rheum. Dis. *26:* 334–340, 1967.

Waaler, E.: On the occurrence of a factor in human serum activating the specific agglutination of sheep blood corpuscles. Acta path. microbiol. scand. *17:* 172–188, 1940.

Acta path. microbiol. scand. Sect. C, *83:* 377–382, 1975

THE BCG-INDUCED RESISTANCE TO LISTERIOSIS

J. BENNEDSEN and S. OLESEN LARSEN

Tuberculosis Department and Biostatistical Department, Statens Seruminstitut, Amager Boulevard 80, DK-2300 Copenhagen S, Denmark

Bennedsen, J. & Olesen Larsen, S. The BCG-induced resistance to listeriosis. Acta path. microbiol. scand. Sect. C, *83:* 377–382, 1975.

BCG immunization schedules for the demonstration of resistance to listeriosis in C3H mice were re-evaluated in a series of experiments. A two-step procedure was found to be superior to a one-step vaccination schedule. Reactivation with BCG five days before challenge caused a significant depression of the multiplication of *Listeria monocytogenes* in the spleen. A saline extract of tubercle bacilli was able to reactivate the BCG immunization in the same way, even though a transient non-immunogenic effect of the same extract was also demonstrated. In the two-step procedure the vaccination dose was found to play a minor role, whereas the reactivation was dose-dependent

Key words: Listeriosis; BCG-induced resistance.

J. Bennedsen, Tuberculosis Department, Statens Seruminstitut, Amager Boulevard 80, DK-2300 Copenhagen S, Denmark.

Received 6.i.75 Accepted 17.iv.75

Listeria monocytogenes (L.m.) infection in mice has been established as a means of studying the resistance to infection with facultative intracellular bacterial parasites (6, 8, 9, 10), especially by *Mackaness* and his colleagues (4, 5, 7, 11, 12, 13, 16). The concept of acquired cellular resistance as an expression of cell-mediated immunity implies that this resistance is specifically evoked but non-specific in action (5). Thus, non-specific resistance to unrelated infections can be used as a measure of cell-mediated immunity, and an influence on the immunity by the addition of specific antigen with the challenge infection can be avoided (2). The duration of the resistance is limited, but reactivation is possible as long as the hypersensitive state is present (2, 3).

In recent years, immunopotentiation has gained interest as a possible tool in the control of infection and tumour growth. In this respect, the BCG-Listeria model is of value as a basis for determination of the degree of monocytic phagocytosis obtainable. The present report describes experiments carried out to re-evaluate the conditions required if resistance against listeria infection in BCG-vaccinated mice is to be achieved. Furthermore, the findings elucidate the reactivation conditions and the dose-responses of immunization and reactivation.

MATERIALS AND METHODS

Animals: 12–16 weeks old male C3H mice, bred at Statens Seruminstitut, Copenhagen.

Microorganisms: The standard BCG vaccine used in most of the experiments was a freeze-dried

routine sample obtained from the BCG Production Department, Statens Seruminstitut. The doses were found to contain approximately 2×10^5 viable units. In some of the experiments, graded doses were given, and the vaccine for that purpose was derived from a concentrated experimental batch of BCG from the same department.

The strain of *Listeria monocytogenes* (SSC 1423) used for challenge was isolated at the Diagnosis Department, Statens Seruminstitut, and kindly supplied by Dr. *H. Lautrop*. The virulence of the strain was maintained by subsequent inoculations into animals, and there was a slight increase in virulence during the experimental period. In the case of C3H mice LD_{50} was estimated to be 0.9×10^4 at the end of the experimental period.

Saline extract of M. tuberculosis: In some of the experiments, a saline extract of *M. tuberculosis* was used for vaccination and reactivation. The preparation of this extract has been reported previously (1).

Immunization and challenge: In all experiments, vaccination, reactivation and challenge were performed by intravenous injection of 0.1 ml in the tail vein. In typical experiments, vaccination was carried out 33 days before challenge and a second injection (called reactivation) was given five days before challenge.

Bacterial counts: Forty-eight hours after challenge, the animals were killed by cervical dislocation. The spleen, and in some cases the liver, of the infected mice was removed aseptically, ground in a mortar, and diluted with distilled water. If any delay in manipulations occurred, cooling in ice was carried out in order to ensure minimal growth of the micro-organisms. Five drops of 0.05 ml of appropriate dilutions were placed on each of two blood agar plates. Counting of the colonies was performed after incubation for 24 h at 37° C and, in some cases, after further incubation in the cold.

Statistical analysis: The mean and the standard deviation of the logarithm of the estimated number of bacteria in each group of animals were calculated. A pooled estimate of the standard deviation 48 h after challenge was $\sigma = 0.8$, being 1 and 24 h after challenge $\sigma = 0.5$. Standard errors of the mean (SE, Table 2) were based on these values.

With a view to comparing the two groups (Table 3), the value $\sigma = 0.8$ was taken to be the true value, being an estimate based on many experiments. A normal distribution u-test was used. In comparisons involving untreated groups, a one-sided test was used whereas a two-sided test was employed in all other cases. As an example, the difference between 7.38 and 5.93 (Expt. A) gives a u-value $= (7.38–5.93)/0.8\sqrt{(1/3 + 1/4)} = 2.37$. Using a one-sided test, this is significant at the 1 per cent level, as denoted in the table by the presence of the pair of symbol †.

TABLE 1. *Death Data on C3H Mice Immunized with BCG 11 Days before Challenge with 30 × LD_{50} of* Listeria monocytogenes

Dose of BCG	Death on day after challenge 2	3	>3	Survivors
–	2	8	0	0
2×10^3	0	10	0	0
2×10^5	1	9	0	0
2×10^7	0	0	4	6

RESULTS

Table 1 shows the survival times of mice vaccinated 11 days before challenge with graded doses of BCG. Only the group of mice given 10^7 v.u. BCG showed significantly prolonged survival times. The challenge doses used brought about the death of all non-immunized mice within three days. Table 2 shows the content of *L.m.* in the spleen of the mice after 1, 24 and 48 h. With a view to comparing different time schedules for the vaccination procedure, it was not found to be of additional value if the increase in spleen counts rather than the values at 48 h, were compared, probably because different animals were involved at the various times.

As basis for subsequent comparisons, the spleen count of *L.m.* 48 h after challenge of normal mice was compared with that of mice which had recovered from a listeria infection 41 to 81 days earlier (Table 3, Expts A and A_1). The difference in spleen count was found to be significant in all cases. Mice vaccinated with BCG 50 days before challenge showed no decrease in *L.m.* content (Expt A).

The time schedule of the protection afforded by BCG vaccination in mice challenged with *L.m.* was examined in Expt B. Under the given conditions, viz. one vaccination dose of 10^5 BCG and a standard challenge dose of about 10^5 *L.m.*, only the group of mice vaccinated 33 days before challenge had a significantly lower count of *L.m.* in the spleen.

TABLE 2. *Spleen Counts (Log Values) at 1, 24 and 48 h after Challenge with* Listeria monocytogenes

Expt	Days between vaccination and challenge*	Challenge dose	1 h			24 h			48 h		
			Mean	n	SE	Mean	n	SE	Mean	n	SE
I	5	2.4×10^5	3.11	3	0.29	5.89	3	0.29	7.35	3	0.46
II	12	2.4×10^5	3.24	3	0.29	5.85	3	0.29	7.14	3	0.46
III	33	2.4×10^5	2.78	3	0.29	4.94	3	0.29	5.65	3	0.46
IV	52	1.7×10^5	2.48	3	0.29	5.55	3	0.29	7.07	3	0.46
V	12	2.6×10^6	3.86	3	0.29	6.18	3	0.29	6.07	2	0.57
VI	33	4.9×10^6	4.47	3	0.29	6.06	3	0.29	6.37	2	0.57
VII	52	4.9×10^6	3.88	2	0.35	7.46	3	0.29	8.18	3	0.46
I, II, III	Unvaccinated controls	2.4×10^5	3.36	8	0.18	5.74	9	0.17	7.26	9	0.27
IV		1.7×10^5	2.89	3	0.29	5.78	3	0.29	7.03	3	0.46
V		2.6×10^6	4.57	3	0.29	5.79	3	0.29	8.12	3	0.46
VI, VII		4.9×10^6	4.61	6	0.20	7.12	6	0.20	8.09	6	0.33

* Vaccination dose for vaccinated groups 2×10^5 BCG.

TABLE 3. *Spleen Counts at 48 h after Challenge with* Listeria monocytogenes *with Various Combinations of Vaccination and Reactivation*

Experiment/ challenge	Vaccination	Days between vaccination and challenge	Reactivation 5 days before challenge	Number of mice	Mean count log value	Difference considered significant P less than: § = 5 per cent, † = 1 per cent, $ = 0.1 per cent																	
A 1.8×10^5	–	–	–	3	7.38	$	†																
	BCG 2×10^5	50	–	3	7.13			$	§														
	LIS 3×10^4	41	–	4	4.72	$		$		§													
	LIS 3×10^3	47	–	4	5.93		†		§	§													
A1 3.6×10^5	–	–	–	9	8.25	$																	
	LIS 3.5×10^3	81	–	9	5.99	$																	
B 2.4×10^5 1.7×10^5	–	–	–	9	7.26	†																	
	BCG 2×10^5	5	–	3	7.35		†																
	BCG 2×10^5	12	–	3	7.14			§															
	BCG 2×10^5	33	–	3	5.65	†	†	§	§														
	BCG 2×10^5	52	–	3	7.07				§														
C 2×10^5	–	–	–	3	7.61	§	§	$	$														
	–	–	Saline ext	3	6.42	§				§	§	§											
	–	–	BCG	3	7.52								†	$									
	Saline ext	33	–	3	7.96					§					§	$	$						
	Saline ext	33	Saline ext	3	7.53													†	$				
	Saline ext	33	BCG	3	8.05						§									§	$	$	
	BCG 2×10^5	33	–	3	6.51		§								§					§			§
	BCG 2×10^5	33	Saline ext	3	5.42			$					†			$		†			$		
	BCG 2×10^5	33	BCG	3	4.89				$			§		$			$		$			$	§
D 3×10^5	–	–	–	10	8.21	§																	
	–	–	Saline ext	10	7.45	§																	
E 3×10^3	–	–	BCG 2×10^5	5	7.28	$	§																
	BCG 2×10^3	33	BCG 2×10^5	5	6.58			§															
	BCG 2×10^5	33	BCG 2×10^5	5	5.37	$		§															
	BCG 2×10^7	33	BCG 2×10^5	5	6.21		§																
F 2.4×10^6	BCG 2.7×10^5	33	–	5	6.19	†	$																
	BCG 2.7×10^5	33	BCG 4.8×10^3	5	6.31			†	$														
	BCG 2.7×10^5	33	BCG 4.8×10^5	5	4.73	†		†		†													
	BCG 2.7×10^5	33	BCG 4.8×10^7	5	3.30		$		$	†													

BCG. When the BCG reactivation was included five days before challenge, the increase in resistance was highly significant compared with the effect of vaccination alone. However, an additive effect of vaccination and reactivation independently cannot be excluded on the basis of the results obtained. If a surface extract of tubercle bacilli (saline extract (1)) was used for reactivation, a parallel rise in resistance occurred.

The action of the saline extract may be open to some doubt. The results indicate that the extract is able to act as antigen in the immunized animals, but is not able to evoke cell-mediated immunity when used in the vaccination procedure. However, a transient resistance following administration of saline extract to normal mice can be seen (Expts C and D). This resistance may be explained as a non-specific activation of macrophages. PPD is reported to act in a similar way, possibly as a consequence of its B cell mitogenic function (15). Listeria filtrate is also able to depress listeria multiplication non-immunogenically (14). The effect was not observed in Expt C when saline extract was used both for vaccination and reactivation, possibly because humoral antibodies neutralize the active compound.

The dose responses of BCG (Expts E and F) reveal a difference between immunization and reactivation. If BCG is used for vaccination, there is indication of a maximum response at 10^5 v.u. and a subsequent decrease the higher the dose. In contrast, the effect of BCG reactivation of primed animals is linearly dose-dependent. As the effect of the second dose of BCG is to introduce specific antigen in order to release a cell-mediated reaction, these differences in dose-responses seem to be quite logical.

In general, the two-injection schedule shown in this study might be advantageous when using BCG if the monocytic apparatus is to be stimulated. Firstly, smaller doses of BCG could be used and secondly, it might be possible in the second stage to test compounds for reactivation properties, even though the compounds were not able to evoke cell-mediated immunity by themselves.

REFERENCES

1. *Bennedsen, J.:* Surface immunogens of *Mycobacterium tuberculosis* demonstrated by immunofluorescence. Acta path. microbiol. scand. Sect. B, *78:* 701–710, 1970.
2. *Blanden, R. V., Lefford, M. I. & Mackaness, G. B.:* The host response to Calmette-Guerin Bacillus infection in mice. J. Exp. Med. *129:* 1079–1107, 1969.
3. *Coppel, S. & Youmans, G. P.:* Specificity of the anamnestic response produced by *Listeria monocytogenes* or *Mycobacterium tuberculosis* to challenge with *Listeria monocytogenes.* J. Bact. *97:* 127–133, 1969.
4. *Mackaness, G. B.:* Cellular resistance to infection. J. Exp. Med. *116*· 381–406, 1962.
5. *Mackaness, G. B.* The immunological basis of acquired cellular resistance. J. exp. Med. *120:* 105–120, 1964.
6. *Mackaness, G. B. & Blanden, R. V.:* Cellular immunity. Progr. Allergy *11:* 89–140, 1967.
7. *Mackaness, G. B.:* The influence of immunologically committed lymphoid cells on macrophage activity in vivo. J. Exp. Med. *129:* 973–992, 1969.
8. *Mackaness G., B.:* Cellular immunity in: van Furth, R.: Mononuclear phagocytes, Blackwell scientific publications, Oxford and Edinburgh, 1970, p. 461–475.
9. *Mackaness, G. B.:* Cellular immunity. Ann. Inst. Pasteur. *120:* 428–437, 1971.
10. *Mackaness, G. B.:* Resistance to intracellular infection. J. Inf. Dis. *123:* 439–445, 1971.
11. *Mackaness, G. B. & Hill, W. C.:* The effect of anti-lymphocyte globulin on cell-mediated resistance to infection. J. exp. Med. *129:* 993–1012, 1969.
12. *North, R J.:* Cellular kinetics associated with the development of acquired cellular resistance. J. exp. Med. *130.* 299–314, 1969.
13. *North, R. J* · The relative importance of blood monocytes and fixed macrophages to the expression of cell-mediated immunity to infection. J. exp. Med. *132:* 521–534, 1970.
14. *Petit, J. C. & Unanue, E R.:* Effects of bacterial products on lymphocytes and macrophages: their possible role in natural resistance to listeria infection in mice. J. Immunol. *113:* 984–992, 1974.
15. *Sultzer, B. M. & Nilsson, B. S.:* PPD tuberculin—a B-cell mitogen· Nature (New Biol.) *340:* 198–200, 1972.
16. *Tripathy, S. P & Mackaness, G. B.:* The effect of cytotoxic agents on the passive transfer of cell-mediated immunity. J Exp. Med. *130. 17–30, 1969.*

Acta path. microbiol. scand. Sect. C, 83: 383-389, 1975

HETEROGENEITY OF PHAGOCYTIC MALFUNCTION IN MYELOID LEUKAEMIA

Localization of the Primary Defect to Decreased Intracellular Killing by Immature Myeloid Cells

CHRISTIAN KOCH

The University Clinic for Infectious Diseases and Statens Seruminstitut,
Department of Clinical Microbiology, Blegdamshospitalet, Copenhagen, Denmark

Koch, Chr. Heterogeneity of phagocytic malfunction in myeloid leukaemia. Localization of the primary defect to decreased intracellular killing by immature myeloid cells. Acta path. microbiol. scand. Sect. C, 83: 383-389, 1975.

The capabilities of circulating leucocytes from 7 patients with chronic myeloid leukaemia (CML) and 3 patients with acute myeloblastic leukaemia (AML) to ingest and to kill *Staphylococcus aureus in vitro* were investigated. Defects in both of these two functions could be detected isolated or in combination. If these functional alterations were correlated with the morphology of the myeloid cell population, the most prominent finding was a highly significant correlation between decreased intracellular killing and degree of admixture of myelocytes and metamyelocytes to the mature cells. The data suggest that the main *qualitative* functional defect in CML as well as in AML is the inefficient intracellular killing capacity displayed by myelocytes and metamyelocytes. All, but one patient, were without signs of bacterial infection at the time of testing. The data thus add to the phenomena previously observed in this laboratory concerning the killing defect in severely infected patients, a defect which was found to be correlated in part with the degree of "shift to the left" of the myeloid cell population.

Key words: Myeloid leukaemia; phagocytic malfunction; decreased intracellular killing.

Chr. Koch, Blegdamshospitalet, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark.

Received 15.v.75 Accepted 29.v.75

A decrease in intracellular killing of *Staphylococcus aureus* by circulating neutrophil granulocytes from patients with bacterial infections has recently been demonstrated (14, 19). In one of these studies (14), a significant correlation between this defect and the "shift to the left", i.e. admixture of less mature cells to the circulating myeloid population, was demonstrated and it was suggested that the defect might in part be caused by malfunction of a subpopulation of immature cells.

The present study explores this question in greater detail by measuring the killing capacity of leucocytes from patients with myeloid leukaemia and varied degrees of admixture of immature cells to the circulating population.

The method employed provides a sensitive measure of the myeloperoxydase-mediated killing capacity in a controlled reaction system of leucocytes and *Staph. aureus*. If the

TABLE 1. *Pertinent Clinical and Laboratory Data on t'*

Pt.	Diagn.	Test	Phase	Treatment	Fever	Infection	Total WBC-count, per µl
1	AML	1	crisis	none	absent	absent	28,400
–	–	2	beginning remission	cytostat.	low-grade	absent	5,900
–	–	3	remission	none	absent	absent	6,800
2	CML	1	active	cytostat. steroids	low-grade	absent	20,000
–	–	2	active	cytostat. steroids	low-grade	absent	29,000
3	CML	1	active	cytostat. steroids	absent	absent	27,800
–	–	2	active (leucopenic-drug-induced?)	steroids	low-grade	present† (urinary-tract)	2,300
–	–	3	active	cytostat. steroids	low-grade	present† (urinary-tract pneumonia?)	?8
4	CML	1	remission	steroids	absent	absent	7,400
–	–	2	remission	steroids	absent	absent	8,000
5	CML	1	active	?	?	?	28,200
6	CML	1	active	cytostat.	absent	absent	25,000
7	AML	1	crisis	(cytostat. withdrawn 8 days prior to test)	absent	absent	5,400
8	CML	1	active	cytostat. steroids	absent	absent	16,000
9	CML	1	active	cytostat.	absent	absent	8,300
10	AML	1	partial remission	steroids	absent	absent	9,400

'or explanation of ingestion- and killing-ratios: see text, methods section
Tests done with 2.5×10^6 total myeloid cells instead of 2.5×10^6 mature (segmented and band-forms) myeloid cells per ml reaction system.

concentration of mature myeloid cells is kept constant (segmented and band-forms), deviations from normal function will be caused by contaminating immature cells and/or malfunction of morphologically mature cells. By way of simultaneous recording of ingestion, the method discriminates between alterations in the individual functions of ingestion and of killing.

METHODS

Ingestion and intracellular *killing* of *Staph. aureus* 502 A were recorded as described previously (14). Briefly, 2.5×10^6 polymorphonuclear leucocytes

Patients Together with Results of Phagocyte Function Studies

Distribution of myeloid cells			Phagocyte function studies			
Blasts to promyelocytes	Myelocytes to metamyelocytes	Band-forms to segmented	Killing-ratio	Ingestion-ratio	Surviving CFU§; Pt./Co. Total	Intracellular
86.3	11.3	2.4	0.57	0.18	61.7	0.31*
44.4	31.6	24.0	0.25	0.43	6.2	1.73*
0	0	100.0	1.38	1.06	1.14	0.77
35.1	29.8	35.1	0.054	0.62	5.47	11.2
14.3	57.1	28.6	0.036	0.85	4.89	23.8
1.2	37.8	61.0	0.27	0.86	5.73	3.12
23.0	43.5	33.3	0.11	0.92	3.86	6.10
?	?	?	0.34	0.67	4.68	1.99
0	0	100.0	0.53	1.15	1.27	2.19
0	0	100.0	0.50	0.47	1.45	0.96
3.1	43.9	53.0	0.07	0.33	2.92	4.63
5.3	32.0	62.7	0.10	1.02	3.9	10.7*
4.6	29.2	66.1	0.058	0.24	4.72	4.10
0	13.1	86.9	0.68	0.42	2.61	0.62
1.2	29.6	69.1	0.21	0.67	1.98	3.23
0	6.2	93.8	0.35	0.43	1.60	1.20

† See text, patients.
§ CFU = colony-forming units *Staph. aureus.*
δ ? denotes imcomplete data at the time of testing.

(segmented and band-forms) were incubated at 35° C during rotation, with an equal number of colony-forming units (CFU) bacteria, in medium containing 10 per cent pooled normal human serum. In a few exceptional cases (noted in Table 1 and Figures), 2.5 × 10^6 total myeloid cells were used, due to scarcity of mature cells. Total CFU, intracellular CFU, and intracellular CFU in the presence of 10 mM sodium azide (NaN_3) which blocks intracellular killing (13), were recorded after 1½ and 3 hours' incubation. *Ingestion* by patient cells is expressed as the ratio: intracellular CFU in NaN_3-blocked cells over the corresponding value in control cells from normal healthy adults, tested simultaneously. *Killing* by patient cells is expressed as the ratio: intracellular CFU in NaN_3-

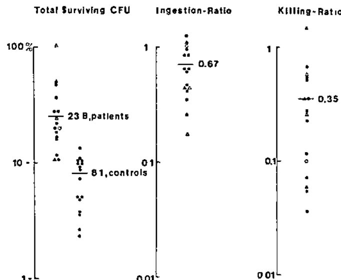


Fig. 1. Graphic presentation of the results of phagocyte function studies, given in Table 1. ●: CML. ▲: AML. Corresponding open symbols: tests done with 2.5×10^6 total myeloid cells per ml reaction system; not included in mean values. Note log-scale.

blocked, divided by intracellular CFU in unblocked cells over the corresponding values for normal control cells.

All values given are those obtained after 3 hours' incubation.

PATIENTS

Seven patients with chronic myeloid leukaemia (CML) and 3 patients with acute myeloblastic leukaemia (AML) were studied, some being studied on several occasions. Pertinent clinical and laboratory data are given in Table 1 together with the results of the functional studies. The patients and times of testing were purposely selected to provide samples of leucocytes representing various combinations of morphologically mature and immature cells. The single criterion for exclusion from the study was the presence of severe bacterial infection. Although several patients ran a low-grade fever, the presence of infection could only be established in one, pt. 3, who suffered from subchronic urinary tract infection with nephropathia and was in long-term treatment with antibiotics at times of testing. Pneumonia was suspected, but not verified, at the time of the third testing. In one patient, pt. 5, clinical data and details of treatment were incomplete. The differential counts were done on the blood samples used for functional studies, with one exception (3rd test in pt. 3).

Statistical calculations Spearman's correlation coefficient R (8).

RESULTS

Fig. 1 shows that the overall number of surviving bacteria in the reaction system was increased, as compared with normal cells, by leucocytes from the patients. This decrease in overall inactivation of bacteria by patient cells could be caused by ineffective ingestion and/or ineffective intracellular killing. Fig. 1 shows that degrees of impairment in both functions varied. In Fig. 2, the ingestion and the killing activities observed in each assay in the patients are plotted against each other. It appears that there is no direct correlation between impaired ingestion and impaired killing. Although several patients exhibited a combination of defects in both functions, isolated defects in either function could be demonstrated in others.

The data were next analysed by correlating the degree of impairment of each function with the morphological composition of the myeloid cell population. There was no strict correlation between the degree of decreased ingestion and the proportion of immature cells whether these were defined as younger than band-forms, younger than metamyelocytes, or younger than myelocytes. However,

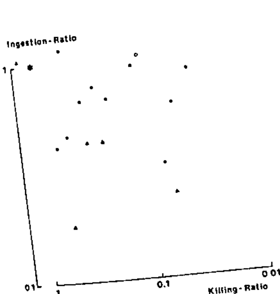


Fig. 2. Relationship between ingestion- and killing-ratio in each test. ●: CML. ▲: AML. Corresponding open symbols: tests done with 2.5×10^6 total myeloid cells per ml reaction system. * denotes normal function. Note log-scale.

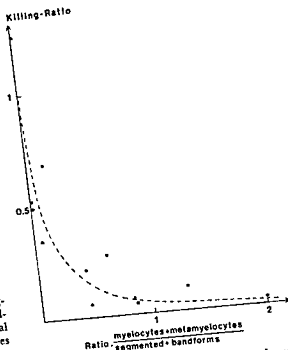


Fig. 3. Relationship between killing-ratio and proportion of "contaminating" immature myeloid cells. All tests are done with 2.5×10^6 mature (segmented and band-forms) myeloid cells per ml reaction system, corresponding to control cells. ●: CML. ▲: AML. Spearman's correlation coefficient R = −0.9161; p<0.001. Note graphic scale.

when the degree of decreased killing was plotted against the ratio of myelocytes and metamyelocytes to mature cells, the correlation was found to be highly significant, as shown in Fig. 3. The correlation coefficient decreased to R = −0.7185 if the ratio of myelocytes, metamyelocytes, and band-forms to segmented was correlated, indicating that myelocytes and metamyelocytes primarily contributed to the decreased killing.

Ingestion and killing were finally correlated with the total and intracellular surviving CFU in the reaction system. No significant correlation between ingestion and either total or intracellular surviving CFU could be detected. As shown in Fig. 4, however, the decreased killing correlated significantly, not only with intracellular surviving CFU, as expected since this value is used for calculation of killing ratio, but also, though less significantly, with total surviving CFU.

DISCUSSION

The present data show that admixture of immature myeloid cells to the mature cell population in the patients caused a marked decrease in intracellular killing (Table 1 and Fig. 3), which was dissociated from co-existing ingestion defects (Fig. 2). A likely interpretation is that myelocytes and metamyelocytes ingested part of the bacteria, but that killing was grossly retarded in these cells. The data shown in Fig. 4 indicate that this defect quantitatively was more important than any co-existing ingestion defect in determining the overall inactivation of the bacteria.

In studies of leucocytes from leukaemic patients, however, the functional changes in morphologically mature as well as immature cells has to be considered. Thus, in CML, there is a gradual decrease in ingestion with increasing immaturity (12, 21) while mature cells displayed normal (12) or decreased (4, 18) ingestion. The latter discrepancy, and the poor correlation between maturity and in-

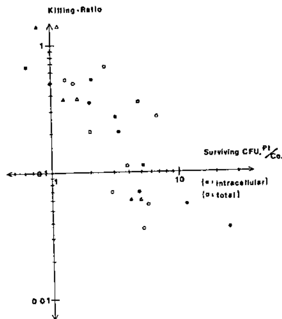


Fig. 4. Relationship between killing-ratio and surviving colony-forming units (CFU) *Staph. aureus.* All tests are done with 2.5 × 10^6 mature myeloid cells per ml reaction system. ●: CML. ▲: AML. Closed symbols: intracellular surviving CFU,— Spearman's correlation coefficient R = −0.9341; $p<0.001$. Open symbols: total surviving CFU,— R = −0.7582; $0.001<p<0.01$. Note log-scale.

gestion in the present studies, however, may be explained by the existence, in CML, of mixtures of populations of active and less active mature cells (3, 16), the ingestion activity in turn being linked to alkaline phosphatase content in the individual cells (17).

Likewise, previous observations of functional (7, 10, 15, 20), ultrastructural (22), and biochemical (5, 9, 15, 20) abnormalities, suggest that killing in mature CML and AML neutrophils is defective. These abnormalities, however, have been seen only in some patients, or in subpopulations of cells in individual patients (5, 7, 15, 22), and in some studies an effect caused by a small fraction of contaminating immature cells should possibly be considered (10, 15, 20).

Since the present studies point towards a major killing defect displayed by immature leukaemic cells, it is interesting that normal killing of *Diplococcus pneumoniae* by immature CML leucocytes has been found by others (12), but a possible explanation may be found in the nature of the test-organism. Unlike *Staph. aureus, D. pneumoniae* can be inactivated in leucocytes from patients with the genetic leucocyte defect: chronic granulomatous disease (CGD), possibly due to substitution of the defective H_2O_2-production displayed by CGD leucocytes, with H_2O_2 produced by *D. pneumoniae* reacting with the intact peroxydase of the CGD cells. In spite of H_2O_2-production, *Staph. aureus* survive in CGD cells due to inactivation by endogenous bacterial katalase (11), thus emphasizing that a killing defect need not involve the handling of all microorganisms to the same extent.

A defect in H_2O_2-production by immature CML leucocytes is thus suggested and indirect evidence of this is provided by the absence of nitroblue-tetrazolium (NBT)-reduction in CML leucocytes less mature than band-forms (1) since both H_2O_2-production and NBT-reduction are linked to oxydative metabolism. There is apparently no quantitative lack of peroxydase in myelocytes and metamyelocytes at least not in the normal (2), but the number of peroxydase-reacting granules has been found to be reduced in neutrophils and their precursors in AML patients, suggesting that several cellular alterations may contribute to the same functional defect (20).

An effect of chemotherapy, in most of the present patients in the form of combination therapy and steroid therapy should also be considered; thus, steroids have recently been shown to depress intracellular killing (6), but it appears from the present study that cellular morphology was more important for functional integrity than the therapeutic regimen applied (Table 1).

The clinical implications of these *in vitro* findings must await studies of homogenous cell populations, or single cells, on the basis of a panel of different microorganisms while the compensatory *in vivo* effect of an increase in the number of circulating myeloid cells should be kept in mind. It might be pointed

out, however, that the substitution with CML cells in leucopenic patients can be expected to be qualitatively inferior to substitution with normal leucocytes obtained by leucophoresis.

The author expresses his gratitude to Professor *V. Faber* and Dr. *Klaus Jensen* for helpful advice and criticism. Mrs. *Ulla Høiby* is thanked for excellent technical assistance. The University Clinic of Internal Medicine C, Gentofte, and A, Rigshospitalet, Copenhagen, Denmark are thanked for placing patients at our disposal for this study. This study was aided by grants from *The Michaelsen Foundation, The Danish Medical Research Council*, grants no. 512-591 -1289 -1940 -3120, and *The Foundation for the Advancement of Medical Science*, Copenhagen, Denmark.

REFERENCES

1. *Ashburn, P., Cooper, M. R., McCall, C. E. & DeChatelet, L. R.:* Nitroblue tetrazolium reduction: false positive and false negative results. Blood *41:* 921–925, 1973.
2. *Bainton, D. F.:* Origin, content, and fate of PMN granules. In: Williams, R. C. & Fudenberg, H. H. (Ed.): Phagocytic mechanisms in health and disease. Georg Thieme Publishers, Stuttgart 1972, pp. 123–136.
3. *Brandt, L.:* Adhesiveness to glass and phagocytic activity of neutrophilic leukocytes in myeloproliferative diseases. Scand. J. Haemat. 2: 126–136, 1965.
4. *Braude, A. I., Feltes, J. & Brooks, M.:* Differences between the activities of mature granulocytes in leukemic and normal blood. J. Clin. Invest. *33:* 1036–1046, 1954.
5. *Catovsky, D., Galton, D. A. G. & Robinson, J.:* Myeloperoxidase-deficient neutrophils in acute myeloid leukaemia. Scand. J. Haemat. 9: 142–148, 1972.
6. *Clemmensen, O., Andersen, V., Hansen, N. E., Karle, H., Koch, C., Søeborg, M. & Weeke, B.:* Sequential studies of lymphocytes, neutrophils, and serum proteins during prednisone treatment. Acta med. scand. Submitted for publication, 1975.
7. *Cline, M. J.:* A new white cell test which measures individual phagocyte function in a mixed leukocyte population. I. A neutrophil defect in acute myelocytic leukemia. J. Lab. Clin. Med. *81:* 311–316, 1973.
8. *Diem, K.* (Ed.): Scientific tables. Geigy, Basle 1962, p. 66.
9. *Gahrton, G.:* The periodic acid-Schiff reaction in neutrophil leukocytes in chronic myeloproliferative diseases. A microspectrophotometric study. Scand. J. Haemat. *3:* 106–116, 1966.
10. *Goldman, J. M. & Th'ng, K. H.:* Phagocytic function of leucocytes from patients with acute myeloid and chronic granulocytic leukaemia. Brit. J. Haemat. 25: 299–308, 1973.
11. *Gray, B. H. & Good, R. A.:* Chronic granulomatous disease of childhood. In: Good, R. A. & Fischer, D. W. (Ed.): Immunobiology. Sinauer Ass. Inc. Publishers, Stamford, Connecticut 1971, pp. 55–61.
12. *Kalinske, R. W. & Hoeprich, P. D.:* Engulfment and bactericidal capabilities of peripheral blood leucocytes in chronic leukemias. Cancer *23:* 1094–1102, 1969.
13. *Koch, C.:* Effect of sodium azide upon normal and pathological granulocyte function. Acta path. microbiol. scand. Sect. B, *82:* 136–142, 1974.
14. *Koch, C.:* Acquired defect in the bactericidal function of neutrophil granulocytes during bacterial infections. Acta path. microbiol. scand. Sect. B, *82:* 439–447, 1974.
15. *Odeberg, H., Olofsson, T. & Olsson, I.:* Granulocyte function in chronic granulocytic leukaemia I. Bactericidal and metabolic capabilities during phagocytosis in isolated granulocytes. Brit. J. Haemat. *29:* 427–441, 1975.
16. *Penny, R. & Galton, D. A. G.:* Studies on neutrophil function II. Pathological aspects. Brit. J. Haemat. *12:* 633–645, 1966.
17. *Pedersen, B. & Hayhoe, F. G. J.:* Relation between phagocytic activity and alkaline phosphatase content of neutrophils in chronic myeloid leukaemia. Brit. J. Haemat. *21:* 257–260, 1971.
18. *Rosner, F., Valmont, I., Kozinn, P. J. & Caroline, L.:* Leukocyte function in patients with leukemia. Cancer *25:* 835–842, 1970.
19. *Solberg, C. O. & Hellum, K. B.:* Neutrophil granulocyte function in bacterial infection. Lancet ii: 727–729, 1972.
20. *Solberg, C. O., Schreiner, A., Hellum, K. B. & Hamre, E.:* Neutrophil granulocyte function in the early diagnosis of acute myelomonocytic and myeloblastic leukaemia. Acta med. scand. *197:* 147–151, 1975.
21. *Strumia, M. M. & Boerner, F.:* Phagocytic activity of circulating cells in the various types of leukemia. Amer. J. Pathol. *13:* 335–349, 1937.
22. *Ullyot, J. L. & Bainton, D. F.:* Azurophilic and specific granules of blood neutrophils in chronic myelogenous leukemia: an ultrastructural and cytochemical analysis. Blood *44:* 469–482, 1974.

BRIEF REPORTS

A RADIOIMMUNOASSAY FOR OCHRATOXIN A: A PRELIMINARY INVESTIGATION

O. Aalund[1], *K. Brunfeldt*[3], *B. Hald*[2], *P. Krogh*[2] & *K. Poulsen*[1]

[1] Laboratory of preventive medicine; [2] Institute of Hygiene and Microbiology, Royal Veterinary and Agricultural University, Copenhagen; [3] The Danish Institute of Protein Chemistry, affiliated to The Danish Academy of Technical Sciences, DK-2970 Hørsholm, Denmark

Aalund, O., Brunfeldt, K., Hald, B., Krogh, P. & Poulsen, K. A radioimmunoassay for ochratoxin A: A preliminary investigation. Acta path. microbiol. scand. Sect. C, *83:* 390–392, 1975.

Ochratoxin A is a nephrotoxic secondary metabolite of several fungal species included in the genera *Aspergillus* and *Penicillium*. A radioimmunoassay for this toxin is described. The reagents included antibodies against a coupling product of ochratoxin A and bovine IgG. ^{125}I-labelled egg-albumin-ochratoxin A was employed as radioactive antigen. Free ochratoxin A dissolved in phosphate-buffered saline inhibited immunoprecipitation of the radioactive antigen. The procedure showed a lower limit of detection at 20 ppb of ochratoxin A.

Key words: Ochratoxin A; radioimmunoassay.

Ole Aalund, Laboratory of preventive medicine, Royal Veterinary and Agricultural University, Bülowsvej 13, DK-1870 Copenhagen V, Denmark.

Received 7.iii.75 Accepted 3.vii.75

Ochratoxin A, a dihydro-isocoumarin derivative linked through its 7-carboxyl group to L-beta-phenylalanine (M.W = 403), is a toxic secondary metabolite of several fungal species included in the genera *Aspergillus* and *Penicillium* (Review: *Chu* 1974). This mycotoxin has been observed as a major disease determinant of mycotoxic nephropathy, a spontaneously occurring renal disease in pigs and other farm animals (Review: *Krogh* 1974). As ochratoxin A contaminated food may reach the human food channel, a comparable renal disorder might exist in man as well. Further elucidation of this possibility requires methods by which low concentrations of ochratoxin A can be detected in small samples of blood, urine, kidney biopsy etc. Thin-layer chromatographic methods commonly used in food analysis are unfit because large samples (50 g) are required. In order to meet the demand for methods applicable to clinical investigations this study was initiated.

Chrystalline ochratoxin A, obtained from fungal cultures of *Aspergillus ochraceus*, was covalently coupled to electrophoretically pure bovine IgG using 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (*McGuigan* 1968). A minute amount of precipitate was formed during the coupling process. Phenylalanine analysis revealed that approximately 20 per cent of the ochratoxin A was coupled to IgG if a 1.2 ochratoxin A/IgG (w/w) was employed in the reaction mixture. The precipitate formed during the coupling procedure was emulsified in complete Freund's adjuvant for intracutaneous injection into the suprascapular region in rabbits. Three rabbits were given 3 initial injections at intervals of 10 days followed by monthly injections. The antigen dose given by each injection was 1.0 mg conjugate protein. The rabbits were bled 10 days after the 3rd initial injection and subsequently 10 days after each monthly injection. All 3 rabbits produced anti ochratoxin A antibodies, but in one of the rabbits this production exceeded by far that in the 2 other rabbits. The antisera produced were used in the radioimmunoassay employing ^{125}I-egg albumin-ochratoxin A as the radioactive antigen. Ochratoxin A had been

Fig. 1 Titration curve. Abscissa: Reciprocal titre. Ordinate (left): Counts per minute (cpm). Ordinate (right): Binding of the radioactive antigen. Maximum immunoprecipitation was approximately 20 per cent of the dose of radioactive antigen employed in the test and this was set to equal 100 per cent in the relative scale for immunoprecipitation. The horizontal line under the titration curve indicates the level of radioactivity in the control system without anti ochratoxin A antibodies.

Fig. 2. Inhibition curve. Abscissa: ng free ochratoxin A added. Ordinate (left): Counts per minute (cpm). Ordinate (right): See legend for Fig. 1. One hundred per cent is the binding to be observed in the absence of free ochratoxin in the system. The horizontal line under the inhibition curve indicates the level of radioactivity in the control system without anti ochratoxin A antibodies. The horizontal line at the top of the figure indicates the level of radioactivity in situations where a placebo inhibitory dose of diluent is used.

coupled to egg albumin according to the procedure described above Chloramine T was used as oxidizing agent in the iodination procedure (*Hunter & Greenwood* 1962) according to the procedure of *McGuigan & Trudeau* (1970). The conjugate in an amount containing 1.4 microgram ochratoxin A was iodinated with 1 mCurie of ^{125}I. Surplus of free ^{125}I was separated from the iodinated conjugate by gel filtration on Sephadex G-50. The iodine uptake was approximately 45 per cent.

The antibody titration curve is shown in Fig. 1. The antiserum dilutions were allowed to react with the ^{125}I-egg albumin ochratoxin A complex for 18 h at 4° C. The diluent used in the test was phosphate-buffered saline (0.15 M NaCl, 0.01 M PO_4, pH 7.0) containing 2 per cent (v/v) inactivated (56° C for 30 min) serum from normal rabbits and 4 per cent (w/v) egg albumin. Following the reaction, goat anti rabbit IgG was added and the mixture was allowed to stand for 4 h at 37° C. After centrifugation, the supernatant was aspirated and the radioactivity of the precipitate was estimated via counting for 10 min in a sodium iodide well type scintillation counter. The reciprocal titre obtained was approximately 12500. The same standard concentration of goat anti rabbit IgG was used in all dilutions of the rabbit anti ochratoxin A antiserum. Also the concentration of normal rabbit serum was the same in all dilutions of the anti ochratoxin A antiserum thus resulting in higher concentration of rabbit IgG in the beginning than in the end of the dilution series.

The binding between the ^{125}I-egg albumin-ochratoxin A complex could readily be inhibited by free ochratoxin A (Fig 2). The anti ochratoxin A antiserum was employed in a 200 times dilution and ochratoxin A was allowed to react with the antiserum for 18 h at 4° C prior to the addition of ^{125}I-egg albumin ochratoxin A.

One more incubation was then performed. The reaction mixture was left to stand for 18 h at 4° C. Using a total volume of 1.125 ml, a minimum amount of approximately 2 ng free ochratoxin A was required for inhibition. The degree of inhibition was found to vary from approximately 9 per cent to approximately 55 per cent, the inhibitory doses ranging from 2 ng to 100 ng of ochratoxin A applied in 0.1 ml of diluent. This might indicate that the procedure has a lower limit of detection at 20 ppb. The only moderate slope of the inhibition curve may be due to a relatively low affinity of free

ochratoxin A for the antibodies synthesized in response to the ochratoxin A-IgG couplmg product.

Work is in progress to assess the potentiality of the procedure for determination of ochratoxin A in biological fluids. Preliminary experiments have demonstrated interference of serum components.

Supported by the *Danish International Development Agency* (*DANIDA*) and the *NOVO Foundation.*

References: Chu, F. S.: Critical Reviews in Toxicology *2:* 499–524, 1974.—*Hunter, W. M. & Greenwood, F. C.:* Nature *194:* 495–496, 1962.—*Krogh, P.:* In: Purchase, I. F. H. (Ed.) Mycotoxins. Elsevier, Amsterdam, 1974, p. 419–428.—*McGuigan, J. E.: Gastroenterology 54:* 1005–1011, 1968.—*McGuigan, J. E. & Trudeau, W. L.:* Gastroenterology *58:* 139–150, 1970.

Acta path. microbiol. scand. Sect. C, 83: 393–396, 1975

THE CELLULAR RESPONSE IN REGIONAL LYMPH NODES AND THYMUS TO REPEATED APPLICATION OF OXAZOLONE TO THE SKIN

Andreas O. Myking

The Gade Institute, Department of Pathology, University of Bergen, N-5000 Bergen, Norway

Myking, A. O. The cellular response in regional lymph nodes and thymus to repeated application of oxazolone to the skin. Acta path. microbiol. scand. Sect. C, 83: 393–396, 1975.

The response of draining lymph nodes, thymus and skin to the repeated application of oxazolone was studied in mice by autoradiography, histology and the recording of organ weights. Transient paracortical hyperplasia was seen in the lymph nodes accompanied by rapid weight loss and depletion of cortical cells in the thymus. With regression of the paracortical hyperplasia, long-lasting hyperplasia of germinal centres and medullary cords developed, while the thymus increased in weight with repopulation of the cortex. The findings suggest some kind of active control or inhibition of the paracortical reaction as the decline was observed during continuous oxazolone application.

Key words: Oxazolone application; cellular response; regional lymph nodes; thymus.

Andreas O. Myking, The Gade Institute, Department of Pathology, University of Bergen, N-5000 Bergen, Norway.

Received 3.vi.75   Accepted 10.vii.75

The cellular events in regional lymph nodes following a single application of a skin sensitizer such as 2-phenyl-4 etoxy methylene-5 oxazolone (oxazolone) have been thoroughly studied (2, 3, 9, 11, 12). Apart from 2 recent reports (8, 10), the effect of several applications of a skin sensitizer on the lymphoid tissue has received little attention.

The present paper reports the preliminary results of cellular changes in draining lymph nodes and thymus following repeated application of oxazolone to mouse skin.

Material and Methods

Animals. Female mice, C3H/A/BOM, 5–6 months old, were used.

The mice were randomized and treated as outlined in Table 1. A total of 0.1 ml oxazolone solution was applied to the skin of both ears and the lower part of the unshaved abdomen (10 per cent dissolved in concentrated ethanol at 40° C). Control animals were treated correspondingly with the diluent. Two μc/g body weight of 3H-thymidin were injected intraperitoneally 2 hours prior to sacrifice of the animals with ether.

Examinations. The body weights were recorded daily throughout the experiment.

The inguinal and auricular lymph nodes, the thymus and the painted skin were fixed in 4 per cent buffered formalin for histological and autoradiographic examination. The organs were weighed after dehydration in ascending concentrations of alcohol and before further processing to toluol and paraffin. The sections were cut at 5 μ and stained with Giemsa and Methylgreen-pyronine. Sections for autoradiography were covered with film strips (Kodak AR 10 Autoradiographic Plates) and developed after 6 weeks of exposure at 4° C. They were stained with haematoxylin through the film. Separate counts of labelled cells in proportion to unlabelled were carried out in paracortex, cortex without germinal centres, germinal centres and medullary cords of lymph nodes and in cortex and medulla of the thymus. A total of 500–1000 cells were counted for each estimate, depending on the concentrations of labelled cells.

TABLE 1. *Schedule for the Experiment with Application of a Concentrated Oxazolone Solution (10 per cent)*

Days of the experiment	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Days on which oxazolone application was performed (▲)	▲			▲				▲			▲				▲			▲				
Number of mice sacrificed																						
Experimental	2		2		2			2		2		2			2							2
Control	2		2		2			2		2		2			2							2
Total number of oxazolone applications	1		1		1			2		3		3			4							6
Interval from last painting to death (days)	2 h		2		4			4		2		4			4							4

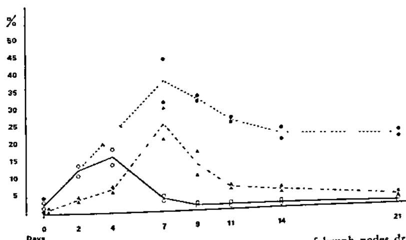


Fig. 1. Proportions of labelled cells (in percentage) in different areas of lymph nodes draining skin sites exposed to repeated application of oxazolone (10 per cent in concentrated ethanol). Each point gives the mean value from the counts in one animal.

———————— ○ paracortex

—·—·—·—·— ▲ cortex

- - - - - - - - - - ● germinal centres

Results

Autoradiography. Fig. 1 illustrates the main findings from the counts in lymph nodes of oxazolone painted mice. The percentage of labelled cells in the T-cell areas showed a peak on day 4, during the next 3 days a fall to day 0 levels which were maintained during the rest of the experiment. The B-cell areas showed the highest ratio on day 7 and thereafter the labelling in the germinal centres showed a gradual fall to a level distinctly higher than that of the starting point. Although no germinal centres were seen on days 2 and 4, the percentage of labelled cortical cells increased during this period.

In the thymus (Fig. 2), the highest counts were recorded on day 7 or somewhat after the peak in the T-cell areas of the lymph nodes.

Weights. The lymph node/body weight ratios increased over 4 times from day 0 to day 9 (Fig. 3). From this day there was a levelling off of the curve

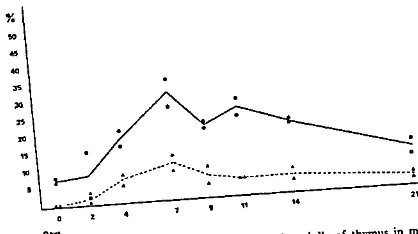


Fig. 2. Proportions of labelled cells (in percentage) in cortex and medulla of thymus in mice exposed to repeated applications of oxazolone (10 per cent in concentrated ethanol) to the skin. Each point gives the figure from the counts in one animal.
———— ● cortex
- - - - - - ▲ medulla

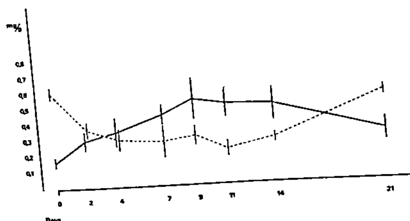


Fig. 3. Variation in weight for thymus and lymph nodes draining skin sites exposed to repeated application of oxazolone (10 per cent in concentrated ethanol), expressed as organ weight/body weight ratios (mg/g). The curve gives the mean value per day. The vertical bars denote the standard deviation (±).
———— lymph nodes
- - - - - - thymus

and a slight decrease during the rest of the experiment. The thymus/body weight ratios decreased from about 0.6 to 0.2 until day 11. Thereafter the weights increased and reached the level of the starting point on day 21.

Histology. The histological findings corresponded closely to the autoradiographic and weight results. Hyperplasia of the paracortical areas with increasing numbers of pyroninophilic blast cells were seen until day 4. Thereafter there was marked germinal centre development together with hy[illegible] of the medullary cords which contained tightly packed plasma cells. The paracortical hyperplasia declined, likewise the number of pyroninophilic blast cells in the paracortex.

The thymus showed depletion of cortical cells as early as day 2. This was marked by days 7, 9 and 11. Thereafter the number of cortical cells increased. On day 21, the histological picture was similar to that of the sham treated controls.

The painted skin revealed small areas of degeneration and necrosis of the epidermis, observed

as early as day 1. Acute inflammatory cells collected at the dermo-epidermal junction during the next 3 days. By the end of the first week, mainly mononuclear cells were present.

Controls. No reaction to the application of the diluent was seen in lymph nodes, thymus and skin. The number of labelled cells in the different areas of lymph nodes corresponded to the figures applying to the experimental animals on day 0. Moderately developed germinal centres with higher concentrations of labelled cells were occasionally observed, especially in inguinal lymph nodes. This occurred independent of the number of ethanol applications.

Discussion

The hyperplasia seen in the paracortex of lymph nodes draining skin sites painted with oxazolone has been shown to be an immunological reaction specific to the sensitizer (12, 13). Systematic studies of the specificity of the hyperplasia seen in the B-cell areas have not been carried out. The interpretation of the B-cell response is difficult partly because concentrated solutions of oxazolone cause extensive necrosis of the epidermis, partly due to lack of methods for the detection of a humoral response to oxazolone. However, it is possible to reproduce in draining lymph nodes the cellular changes here described with low concentrations that leave the skin without damage (7). This lends support to the assumption that also the hyperplasia of the B-cell areas is generated specifically by oxazolone.

An interesting point in this experiment is the decline of the hyperplasia in the T-cell areas seen while the prolonged response in the B-cell areas developed. Parallel with this, the initial cellular depletion in the thymus was followed by repopulation of cortical cells. These events took place during continuous oxazolone application and therefore suggest some kind of inhibition or active control of the T-cell response. It has been demonstrated that suppression of the B-cell pool with immunosuppressive agents causes increased intensity and prolongation of contact hypersensitivity (14, 15). During certain immunization procedures, cells are generated which suppress delayed hypersensitivity reactions. The suppression has been shown to be immunologically specific and dependent on living cells, most likely B-cells (5, 6). Depression of contact hypersensitivity by serum factors has also been demonstrated (1, 4).

The rapid decline of the paracortical hyperplasia in the draining lymph nodes could be explained along the same lines as those put foreward for the reduced responsiveness of the skin to sensitizing agents. However, whether it depends on the presence of living cells or on humoral factors remains to be shown, likewise that the regression of the hyperplasia in the T-cell areas is due to functions of B-cells. However, this possibility is in good agreement with the long-lasting hyperplasia of the B-cell areas seen to develop while the paracortical reaction declined.

I wish to thank Professor *Olav Tönder* for his interest in the present study and for constructive criticism. I also wish to thank Professor *Flora Hartveit* for reading the manuscript. The skilled technical assistance of Mrs. *Anne Marie Sundström* is gratefully acknowledged. Financial support has been received from *Dr. F. G. Gades Legat*, Bergen.

References: 1. *Asherson, G. L., Zembala, M. & Barnes, R. M. R.*: Clin. exp. Immunol. *9:* 111–121, 1971.—2. *Davies, A. J. S., Carter, R. L., Leuchars, E. & Wallis, V.*: Immunology *17:* 111–126, 1969.—3. *Fjelde, A. & Turk, J. L.*: Nature *205:* 813–815, 1965.—4. *Halliday, W. J. & Walters, B. A. J.*: Clin. exp. Immunol. *16*: 203–212, 1974.—5. *Katz, S. J., Parker, D., Sommer, G & Turk, J. L.*: Nature *248:* 612–614, 1974.—6 *Katz, S. J., Parker, D. & Turk, J. L.*: Nature *251:* 550–551, 1974.—7. *Myking, A. O.*: To be published.—8. *Myking, A. O.*: Acta path. microbiol scand. Sect. A, *82*: 249–254, 1974.—9 *Oort, J. & Turk, J. L*: Brit. J. exp. path. *46:* 147–154, 1965.—10. *Oort, J., Scheper, R. J. & Veldhuizen, R. W.*: In: Parker, D. & Turk, J. L. (Eds.): Contact Hypersensitivity in Experimental Animals. Monographs in Allergy. vol. 8 S. Karger, Basel, 1974, p 136.—11. *Parrott, D. M. V.*: Suppl. J. Clin. Path. *20* 456–465, 1967.—12. *Turk, J. L. & Stone, S. H.* In: Amos, B. & Koprowski, H. (Eds.): Cellbound antibodies. Wistar Institute Press. Philadelphia, 1963, p. 51–60.—13. *Turk, J. L.*: In: Neuberger, A & Tatum, E. L. (Eds.): Frontiers in biology, vol. 4. North Holland Publishing Company, Amsterdam, 1967, p. 143.—14. *Turk, J. L., Parker, D. & Poulter, L. W.*: Immunology *23:* 493–501, 1972.—15 *Turk, J. L. & Parker, D.*: Immunology *24*. 751–754, 1973.

Acta path. microbiol. scand. Sect. C, 83: 397–405, 1975

CROSS-REACTIONS OF *KLEBSIELLA*

*Immunochemical Relationships Indicated by Cross-Reactions in Antipneumococcal Sera and Tested in Anti-*Klebsiella *Sera*

MICHAEL HEIDELBERGER*, WOLFGANG NIMMICH**, JORUNN ERIKSEN§, GUY G. S. DUTTON§§, STEPHAN STIRM† and CHYANG T. FANG††

*Dept. of Pathology, New York University Medical Center, New York, NY 10016, U.S.A.
**Institut für med. Mikrobiologie u. Epidemiologie, Universität Rostock, 25 Rostock 1, D.D.R.
§Kaptein W. Wilhelmsen og Frues Bakteriologiske Institutt, Rikshospitalet, Oslo, Norway.
§§Dept. of Chemistry, Univ. of British Columbia, Vancouver, B C., Canada.
†Max Planck Institut für Immunbiologie, 78 Freiburg-Zähringen, W. Germany.
††Present address: Blood Research Laboratory, American National Red Cross, Bethesda, Md. 20014, U.S.A.

Heidelberger, M., Nimmich, W., Eriksen, J., Dutton, Guy G S, Stirm, S. & Fang, C. T. Cross-reactions of *Klebsiella*. Immunochemical relationships indicated by cross-reactions in antipneumococcal sera and tested in anti-*Klebsiella* sera. Acta path. microbiol. scand. Sect. C, *83:* 397–405, 1975.

An earlier study of more than 60 *Klebsiella* K-polysaccharides in anti-pneumococcal sera of many capsular types provided the impetus for the present work. With anti-*Klebsiella* sera, some of these cross-reactions are now shown to be reciprocal. In most instances in which several or many K-substances precipitated antipneumococcal sera of a given type, these same K-polysaccharides were cross-reactive with one or more anti-*Klebsiella* sera belonging to the same group For example, the polysaccharides K1, 15, 24, 31, 39, 42, and 59 precipitated an equine antipneumococcal type X serum. Pneumococcal capsular polysaccharide type X and *Klebsiella* polysaccharides K15, 24, 31, 39, 42, and 59 precipitated one or both of the equine anti-*Klebsiella* K1 sera available. Similar results with anti-*Klebsiella* rabbit sera to other K-types show the validity of this approach in revealing hitherto unknown serological, and hence chemical, relationships between the K-types

Key words: *Klebsiella;* cross-reactions.

Michael Heidelberger, Dept. of Pathology, New York University Medical Center, New York, NY 10016, U.S.A.

Received 13.vii.75 Accepted 13.vii.75

Recent studies have been made of cross-precipitation of more than 60 *Klebsiella* K polysaccharides in a large number of antipneumococcal (anti-Pn) sera and a few anti-*Salmonella* sera (18). The resulting data were in general agreement with established structures of such *Klebsiella* and pneumococcal polysaccharides of which these features were known. Predictions could also be made of structures which would be found in K polysaccharides of unknown structure showing appropriate cross-reactivities. Many reactions could not be interpreted because of lack of knowledge of the structures of the pneumococcal (S) or *Klebsiella K* polysaccharides involved.

The availability of a number of anti-

Klebsiella sera has made it possible to study cross-reactions in the opposite direction. Although no massive precipitation of these antisera by pneumococcal capsular polysaccharides has as yet been observed, such as occurred frequently with *Klebsiella* K-substances and antipneumococcal sera, the earlier work (18) has nevertheless served as a guide to the discovery of a number of hitherto unobserved cross-precipitations among the *Klebsiella* K-polysaccharides for which the small number of known structures had given few indications. Chemical and microbiological studies now under way in a number of laboratories will test the accuracy of the predictions made. Cross-reactions among the K types of *Klebsiella* have been recorded (10, 24, 25, 26) and the present paper adds greatly to their number and, in some instances, relates cross-reactivity to the chemical structures of the K polysaccharides.

TABLE 1. *Cross-reactions of Pneumococcal and* Klebsiella *Type-specific Polysaccharides in Anti-*Klebsiella *Type 1 Sera*

Polysaccharide	Maximal antibody nitrogen precipitated at 0° C per ml antiserum, μg — Horse 487, 4/17/33	Horse 488, 3/14/34
K1 (homologous)	1140	600
K3 (homologous?)	6	33
PnS X	6	±
S XIV	+	—
S XX	±	12
K15	51[a]	35
K24	75[b]	31
K31	334	228[c]
K39	180	108[d]
K42	+ + ±	+ + ±
K59	+ +	

a Supernatants + K24 gave 66 μg N.
b Supernatants + K15, K39 pptd. 9,14 μg N resp., supernatants after K15 gave 181 μg N with K31, 15 μg N with K39.
c Supernatants from which 217 μg N had been pptd. gave no ppt. with K15, 19 μg N with K39. Antiserum drawn 3/16/34, with 300 μg anti-K1 N/ml, gave 128 μg N with K31. Supernatants + K1 gave 164 μg N. This + 128 pptd. by K31 = 292, showing that K31 ppts. *antibody N*.
d Supernatants from which 85 μg N had been pptd. gave 178 μg N with K31

MATERIALS AND METHODS

Equine anti-*Klebsiella* K 1 sera were supplied by Dr. *Gerald M. Ward, New York City Dept. of* Health Laboratories, Otisville, N.Y. 10963. Anti-K sera were raised in rabbits (R) by co-authors E., H., N., and S. These letters are used for the appropriate sera.

Pneumococcal polysaccharides were, in part, our own, in part those isolated by Dr. *Rachel Brown, New York State Dept. of Health Laboratories,* Albany, N.Y., 12208, and others from Merck, Sharp and Dohme and from Squibb.

Qualitative tests were carried out preferably with 1 ml of antiserum, to which 0.01 or 0.02 mg of polysaccharide was added, usually in 1 mg/ml solution in 0.9 per cent saline. Tests were allowed to stand for 6 to 8 days in a refrigerator or cold room and read after immersion in ice-water. Results are recorded on a scale of — to + + + +. The process was repeated after addition of another 0.01 or 0.02 mg of polysaccharide.

Quantitative micro-analyses of the homologous and cross-reactions were run as in previous papers (21, 22, 23). Homologous precipitations were allowed to stand in a bath at 0° C, with occasional twirling of the tubes, for 2 to 4 days before centrifugation and washing. Cross-reactions were left at 0° C for 4 days to 2 weeks or more, depending upon the speed and intensity of precipitation.

RESULTS AND DISCUSSION

Antisera to Klebsiella *Type K1*

Two equine antisera drawn more than 40 years ago were found. Immunization had been with "Pn Friedlander" and "Friedlander's pneumo, K"* but the serological type was not specified. Tests of various bleedings of the horses, New York City 487 and 488, were set up with polysaccharides K1, 2, and 3. K1 precipitated later bleedings of both horses rapidly and strongly, K2 was negative, K3 gave weak precipitation. Serum 487 of 4/17/33 and 488** of 3/14/34 were selected

* Designations apparently used at the time for *Klebsiella* strains responsible for pneumonia *in human beings* (*cf. Zinnser, H.,* Microbiology, *15" Ed., p. 530*).

** The anti-Pn X-XX equine serum 488 used in (23) was drawn in 1939 at the New York *State* Laboratories.

TABLE 2. *Cross-reactions of Pneumococcal and* Klebsiella *Type-specific Polysaccharides in Anti-* Klebsiella *Types 2, 9, and 51 Sera*

Polysaccharide	Maximal antibody N precipitated at 0° C per ml antiserum, µg			
	RK2_1 (E)	RK2_{33} (H)	RK9 (E)	RK51 (E)
Homologous	208	490	+ + ±	≧205
Pn S II	39[a]	102[g]		74[k]
K2			+	6
K4	11	+		
K8	6[b]	+		
K30	109[c]	310[h]	+ ±	6[l]
K33	88[d]	262		+ ±[l]
K51	53[e]	86[i]		
K55	37[f]	48[j]		
K59	+ +			

[a] Supernatants + K30 gave 67 µg N; intact serum gave 97 at level used.
[b] Not run in duplicate. Supernatant + K45: + ±.
[c] Supernatants pptd. 5 µg N with K51.
[d] Analysis run with supernatants from pptn. with K4; these also gave + ± with K27.
[e] Supernatants + K30 gave 71 µg N; + K33: + + +.
[f] Supernatants + Pn S II, + + ; + K4, ±.
[g] Supernatants + K30, K33 gave 133, 145 µg N, resp.
[h] Supernatants + S II pptd. 13 µg N; supernatants from 256 µg N gave 194 with K2; total 450.
[i] Supernatants from an average of 60 µg N gave 19 µg N with K55. Other supernatants from 84 µg N gave 49 µg N with S II, 178 with K33.
[j] Supernatants + K51, SII pptd. 39, 77 µg N, respectively. Supernatants from the pptn. of K55 followed by K51 gave a further 200 µg N with K30.
[k] Supernatants which had given no ppt. with K9, gave 6 µg N with K30, + ± with K33, and 126 µg N with K51; total, 206 plus + ±, approx. 210.
[l] See footnote k.

as the most potent; these were negative with Pn SI, II, III, IV.

Although K1 precipitated 83 µg of antibody nitrogen per ml of anti-Pn X627 (18) the reciprocal reaction, that of Pn S X (the structure of which is not known) in these antisera, was minimal. The earlier studies had shown that K31, 39, and 59 precipitated 52, 51, and 56 µgN, respectively, from anti-Pn X627, while K15, 22, 24, and 42 gave + + ± qualitative tests. The anti-K1 sera were therefore tested with these K-substances with results shown in Table 1.

Four of the K polysaccharides react strongly in the two antisera, two others appreciably. K31, the structure of which is not own, precipitates more than one-third of e antibody in serum 488 and almost a third that in 487. Footnotes b and c show that is is in large part the same fraction of antibody with which K15, 24, and 39 react, and that the reactive antibody is actually anti-K1. K15, 24, 31, and 39 therefore contain serologically, and hence chemically similar structures, and some of the antisera to these types should show mutual cross-reactivity in one or both directions. It will be recalled that antisera raised in rabbits are often less cross-reactive than equine antisera, (see for example (17)), most likely because the anticarbohydrate of the latter is largely macromolecular (12, 19, 20).

Of the K-substances which cross-react in anti-K1, the structures of only K24 (5) and K59 (30) are known. Both contain 1,3-linked D-glc and D-man linked 1,2- and 1,3-. K1 is made up of D-glcA, D-glc, and L-fucose. Some of the glc, at least, occurs as $[\text{D-Glc-}(1\rightarrow]_n$ and most of the other linkages are believed to be 1,3-(1). Possibly the β-D-man lateral

Klebsiella sera has made it possible to study cross-reactions in the opposite direction. Although no massive precipitation of these antisera by pneumococcal capsular polysaccharides has as yet been observed, such as occurred frequently with *Klebsiella* K-substances and antipneumococcal sera, the earlier work (18) has nevertheless served as a guide to the discovery of a number of hitherto unobserved cross-precipitations among the *Klebsiella* K-polysaccharides for which the small number of known structures had given few indications. Chemical and microbiological studies now under way in a number of laboratories will test the accuracy of the predictions made. Cross-reactions among the K types of *Klebsiella* have been recorded (10, 24, 25, 26) and the present paper adds greatly to their number and, in some instances, relates cross-reactivity to the chemical structures of the K polysaccharides.

TABLE 1. *Cross-reactions of Pneumococcal and* Klebsiella *Type-specific Polysaccharides in Anti-*Klebsiella *Type 1 Sera*

Polysaccharide	Maximal antibody nitrogen precipitated at 0° C per ml antiserum, µg	
	Horse 487, 4/17/33	Horse 488, 3/14/34
K1 (homologous)	1140	600
K3 (homologous?)	6	33
PnS X	6	±
S XIV	+	—
S XX	±	12
K15	51[a]	35
K24	75[b]	31
K31	334	228[c]
K39	180	108[d]
K42	+ + ±	+ + ±
K59	+ +	

a Supernatants + K24 gave 66 µg N.
b Supernatants + K15, K39 pptd. 9,14 µg N resp.; supernatants after K15 gave 181 µg N with K31, 15 µg N with K39.
c Supernatants from which 217 µg N had been pptd. gave no ppt. with K15, 19 µg N with K39. Antiserum drawn 3/16/34, with 300 µg anti-K1 N/ml, gave 128 µg N with K31. Supernatants + K1 gave 164 µg N This + 128 pptd. by K31 = 292, showing that K31 ppts. *antibody N.*
d Supernatants from which 85 µg N had been pptd. gave 178 µg N with K31.

MATERIALS AND METHODS

Equine anti-*Klebsiella* K1 sera were supplied by Dr. *Gerald M. Ward*, New York City Dept. of Health Laboratories, Otisville, N.Y. 10963. Anti-K sera were raised in rabbits (R) by co-authors E., H., N., and S. These letters are used for the appropriate sera.

Pneumococcal polysaccharides were, in part, our own, in part those isolated by Dr. *Rachel Brown*, New York State Dept. of Health Laboratories, Albany, N.Y., 12208, and others from Merck, Sharp and Dohme and from Squibb.

Qualitative tests were carried out preferably with 1 ml of antiserum, to which 0.01 or 0.02 mg of polysaccharide was added, usually in 1 mg/ml solution in 0.9 per cent saline. Tests were allowed to stand for 6 to 8 days in a refrigerator or cold room and read after immersion in ice-water. Results are recorded on a scale of — to + + + +. The process was repeated after addition of another 0.01 or 0.02 mg of polysaccharide.

Quantitative micro-analyses of the homologous and cross-reactions were run as in previous papers (21, 22, 23). Homologous precipitations were allowed to stand in a bath at 0° C, with occasional twirling of the tubes, for 2 to 4 days before centrifugation and washing. Cross-reactions were left at 0° C for 4 days to 2 weeks or more, depending upon the speed and intensity of precipitation.

RESULTS AND DISCUSSION

Antisera to Klebsiella *Type K1*

Two equine antisera drawn more than 40 years ago were found. Immunization had been with "Pn Friedlander" and "Friedlander's pneumo, K"* but the serological type was not specified. Tests of various bleedings of the horses, New York City 487 and 488, were set up with polysaccharides K1, 2, and 3. K1 precipitated later bleedings of both horses rapidly and strongly, K2 was negative, K3 gave weak precipitation. Serum 487 of 4/17/33 and 488** of 3/14/34 were selected

* Designations apparently used at the time for *Klebsiella* strains responsible for pneumonia in human beings (cf. *Zinsser, H., Microbiology*, 15th Ed., p 530).

** The anti-Pn X-XX equine serum 488 used in (23) was drawn in 1939 at the New York State Laboratories.

(34). Cross-reactions of the serotypes 8, 11, 21, 26, and 35 were studied long ago (24), but it is now possible to relate some of the cross-reactivity of anti-K11 to the structures of the polysaccharides involved. The *immunodominant group of K11 is the lateral, 4,6-pyruvylated non-reducing end-group of* D-galactose *in the repeating unit, and this* makes possible the observed reactions with K21 (4, 8) and colanic acid (11, 28) which possess the same end-groups (34). Since these also occur in the capsular polysaccharide of *Rhizobium trifolii* TA_1 (3) precipitation in anti-K11 was expected and found. Cross-reactivity with serotype K8 would seem to be due to the →3)-D-gal-α-(1→3)-D-glc-β-(1— sequence which occurs internally in the main chain of the repeating unit of K8 (33) and *connects the repeating units in K11. The structures of K26 and 35 are not known, nor* are those of K12 and 13, which give small, presently uninterpretable reactions in anti-K11. Possibly some of the weaker precipitations recorded in Table 4 are due to the presence of O3 lipopolysaccharide as a contaminant in the preparations since the serum contains antibody to this substance. K32 gave fairly strong precipitation, but the reason for it is not known; *K32 reacts heavily in anti-Pn IV and SIV has PyA on D-gal, probably attached to positions* 2 and 3 (unpublished data). The exocellular polysaccharide of *Xanthomonas campestris* also precipitates anti-K11 strongly although its PyA is at 4,6-on non-reducing end-groups of D-glc (13, 32). It has -D-glcA-β-(1→2)-D-man- in its main chain (32).

Antiserum to serotype K22 was tested because *the polysaccharides K22, 33 and 43 precipitated anti-Pn XV strongly* (18). The reciprocal cross-reaction of S XV in the single anti-K22 tested was weak but definite (Table 4). K43 also reacted with anti-K22 and K33 precipitated the antiserum fairly heavily (Table 4), so that the capacity of K22, 33, and 43 to react in anti-Pn XV has revealed *immunological and serological relationships among three serotypes of Klebsiella* which had not previously been known to possess antigenic groupings in common. Although K2 also reacted with anti-Pn XV as strongly as did K33, it did not precipitate the anti-K22.

The polysaccharide of serotype K32 precipitated heavily in all anti-Pn IV sera tested (16), but the available anti-K32 failed to *precipitate S IV, nor did it react with K51 which precipitated anti-Pn IV less strongly* (18). Other anti-K32 sera might very well be expected to cross-react with S IV and/or K51.

Another unidirectional cross-reaction was that of K49 in anti-Pn I (18). Neither of the anti-K49 sera tested precipitated S I or K63, which was also reactive in anti-Pn I. Both anti-K49s precipitated K48, which had reacted weakly in anti-Pn I. An anti-K48 precipitated K49 *(Table 4), so that the K48–K49 relationship is a reciprocal one.* Galacturonic acid is the only sugar in common and K49 apparently contains a much larger proportion of D-galA linked similarly to that in S I (14). K48 precipitated anti-Pn XIX and K49 reacted with anti-Pn XXV (18), but the reciprocal precipitations, S XIX anti-K48 and S XXV anti-K49, were *negative to doubtful.*

*Cross-precipitation in Anti-*Klebsiella *Types 19 and 47 (Table 5)*

These were two of the three K types most strongly reactive in anti-Pn XXIII (18). Other good precipitators of anti-XXIII were K14, 34, 49, 56, and 64. *S XXII and K52 were also tested in anti-K47 because most of the Ks which reacted with anti-XXIII also precipitated*, though less strongly, with anti-Pn XXII. Like S XXIII (15) and possibly S XXII (2) (which gives ++ in anti-Pn XXIII), K47 and 56 contain lateral non-reducing end-groups of L-rhamnose which were predicted from *their substantial precipitation of anti-Pn XXIII* (for K47 see [16]). *It is probable that* K19 also *contains* such end-groups, since both K47 and 56 precipitate the rather weak anti-K19 available, as does K17. K17, 19, 49 and 53

TABLE 6. *Cross-precipitation in Anti-*Klebsiella *Types 43 and 57*

Polysaccharide	Maximal antibody N precipitated at 0° C per ml antiserum, μg		
	$RK43_{142}$ (E)	$RK57_{266}$ (E)	RK57 (*N*)
Homologous	171	4200	310
Pn S XVIII	—[a]	0	—
K39		12	9[b]
K43		2890	34
K57	+ + ±		
K62		+	+ +[c]

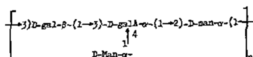
[a] Tested after prior negative with K22, which, like K43, pptd. anti-Pn XV strongly. K22 did not ppt. *anti-Pn XVIII.*

[b] Analyses on supernatants from those for S XVIII and K62.

[c] Tested in part with negative supernatants from S XVIII.

2200. K39 gave 29 μg N, K26 a roughly equivalent + + ±, and K62 —. Although the reverse reaction of S XVIII in the antisera to the two K types thus far tested was negative, K43 and K57 showed a previously unknown close relationship marked by reciprocal cross-precipitation. The reaction in the strong rabbit anti-$K57_{266}$ involved 69 per cent of the antibody precipitable by the homologous polysaccharide.

The structure of K57 is given *(30)* as



K43 contains the same neutral sugars, but its acidic component is glucuronic acid and the linkages are unknown. The massive cross-reaction in anti-$K57_{266}$ may indicate that its mannose is linked similarly to that in K57. K62 was tested because it is said to possess lateral non-reducing end-groups of D-man and 1,3-linked D-gal (7).

K25 is now known to have →4)-D-glc-β-(1→3)-D-man (*H. Niemann & S. Stirm*, personal communication). This might account for its limited reactivity in anti-K5. K37 also has D-glc linked 1→3 to D-gal (29). Cross-reactions of strain K48 in anti-49 and of K55 in anti-51 have been noted as capsular swelling by *M. W. Casewell* (J. Clin. Path. *28:* 33–36, 1975), also K47 in anti-17.

Aided by a grant from the National Science Foundation, U.S.A., with technical assistance by Mr. *Paul Howard.*

REFERENCES

1. *Barker, S. A., Brimacombe, J. S., Eriksen, J. L. & Stacey, M.:* Capsular polysaccharide of *Klebsiella pneumoniae* type A (strain 1265). Nature *197:* 899–900, 1963.
2. *Chattopadhyay, B. P.:* Dissertation. Univ. of Calcutta. 1973.
3. *Chaudhari, A. S., Bishop, C. T. & Dudman, W. F.:* Structural studies on the specific capsular polysaccharide from *Rhizobium trifolii*, TA-1. Carbohydr. Res. *28:* 221–231, 1973.
4. *Choy, Y.-M. & Dutton, G. G. S.:* The structure of the capsular polysaccharide from *Klebsiella* K-type 21. Can. J. Chem. *51:* 198–207, 1973.
5. *Choy, Y.-M., Dutton, G. G. S. & Zanlungo, A. M :* Structure of the capsular polysaccharide of *Klebsiella* K-type 24. Can. J. Chem. *51*· 1819–1825, 1973.
6. *Curtis, S. N. & Krause, R. M.:* Antigenic relationships between groups B and G streptococci. J. Exp Med. *120:* 629–637, 1964.
7. *Dutton, G. G. S.:* Personal communication.
8. *Dutton, G. G. S. & Choy, Y.-M.:* Capsular polysaccharide from *Klebsiella* type 21. Carbohydr. Res. *21:* 169–172, 1972.
9. *Dutton, G. G. S. & Yang, M.-T.:* Structure of the capsular polysaccharide of *Klebsiella* K-type 5. Can. J. Chem. *51:* 1826–1832, 1973.
10. *Eriksen, J.:* 13. Serological investigation of the cross-reaction of *Klebsiella* type 3(C), *Klebsiella aerogenes* strain B 1076/48 and

"*Enterobacter*" strain 349. Acta path. microbiol. scand. *64:* 527–533, 1965.

11. *Garegg, P. J., Lindberg, B., Onn, T. & Sutherland, I. W.:* Comparative studies on the M-antigen from *Salmonella typhimurium*, *Escherichia coli* and *Aerobacter cloacae*. Acta Chem. Scand. 25: 2103–2108, 1971.
12. *Goodner, K., Horsfall, F. L., Jr. & Bauer, J. H.:* Ultrafiltration of type I antipneumococcal sera. Proc. Soc. Exp. Biol. Med. *34:* 617–619, 1936.
13. *Gorin, P. A. J., Ishikawa, T., Spencer, J. F. T. & Sloneker, J. H.:* Configuration of the pyruvic acid ketals, 4,6-O-linked to D-glucose units, in *Xanthomonas campestris* polysaccharide. Can. J. Chem. *45:* 2005–2008, 1967.
14. *Guy, R. C. E., How, M. J., Stacey, M. & Heidelberger, M.:* The capsular polysaccharide of type I Pneumococcus. J. Biol. Chem. *242:* 5106–5111, 1967.
15. *Heidelberger, M., Davie, J. M. & Krause, R. M.:* Cross-reactions of the group specific polysaccharides of streptococcal groups B and G in antipneumococcal sera with special reference to type XXIII and its determinants. J. Immunol. *99:* 794–796, 1967.
16. *Heidelberger, M., Dudman, W. F. & Nimmich, W.:* Immunochemical relationships of certain capsular polysaccharides of *Klebsiella*, pneumococci, and *Rhizobia*. J. Immunol. *104:* 1321–1328, 1970.
17. *Heidelberger, M., Kabat, E. A. & Shrivastava, D. L.:* Quantitative study of the cross-reaction of types III and VIII pneumococci in horse and rabbit antisera. J. Exp. Med. *65:* 487–496, 1937.
18. *Heidelberger, M. & Nimmich, W.:* Immunochemical relationships between bacteria belonging to two separate families: pneumococci and *Klebsiella*. Immunochem. *12*. 1975.
19. *Heidelberger, M. & Pedersen, K. O.:* The molecular weight of antibodies J. Exp. Med. 65 393–414, 1937.
20. *Heidelberger, M., Pedersen, K. O. & Tiselius, A.:* Ultracentrifugal and electrophoretic studies on antibodies. Nature *138:* 165, 1936.
21. *Heidelberger, M. & Rebers, P. A.:* Cross-reactions of polyglucoses in antipneumococcal sera. VI. J. Am. Chem. Soc *80:* 116–118, 1958.
22. *Heidelberger, M. & Rebers, P. A..* Immunochemistry of the pneumococcal types II, V, and VI. J. Bact. *80:* 145–153, 1960.
23. *Heidelberger, M. & Tyler, J. M.:* Cross-reactions of pneumococcal types. J. Exp. Med. *120:* 711–719, 1964.
24. *Henriksen, S. D.:* Studies on the *Klebsiella* group (Kauffmann). IV. Cross-reactions of *Klebsiella* types 8, 11, 21, 26 and 35, and the M-antigen of *Escherichia coli*. Acta path. microbiol. scand. *34:* 271–275, 1954.
25. *Henriksen, S. D. & Eriksen, J.:* Immunochemical studies on some serological cross-reactions in the *Klebsiella* group. 1. Quantitative study of the cross-reactions between Klebsiella types A(1), E(5) and AE. Acta path. microbiol. scand. *45:* 381–386, 1959.
26. *Henriksen, S. D. & Eriksen, J.:* 7. Serological reactions of some strains of type 3(C) and some cross-reacting strains. Acta path. microbiol. scand. *54:* 391–397, 1962.
27. *E.g.*, Kotelko, K., Staub, A. M. & Tinelli, R.: Étude immunochimique sur les *Salmonella*. VIII. Rôle des groupements O acetyls dans la spécificité du facteur 0:5. Ann. Inst. Pasteur *100:* 618–637, 1961.
28. *Lawson, C. J., McCleary, C. W., Nakada, H. I., Rees, D. A., Sutherland, I. W. & Wilkinson, J. F.:* Structural analysis of colanic acid from *Escherichia coli* by using methylation and base-catalysed fragmentation. Biochem. J. *115:* 947–958, 1969.
29. *Lindberg, B.:* Personal communication.
30. *Lindberg, B., Lönngren, J., Rudén, U. & Nimmich, W.:* Structural studies of the capsular polysaccharide of *Klebsiella* type 59. Carbohydr. Res. *42:* 83–93, 1975.
31. *Nimmich, W.:* Zur Isolierung und Qualitative Bausteinanalyse der K-Antigene von *Klebsiellen*. Z. Med. Mikrobiol. u. Immunol. *154:* 117–131, 1968.
32. *Orentas, D. G., Sloneker, J. H. & Jeanes, A.:* Pyruvic acid content and constituent sugars of exocellular polysaccharides from different species of the genus *Xanthomonas*. Can. J. Microbiol. *9:* 427–430, 1963.
33. *Sutherland, I. W.:* Structure of the *Klebsiella aerogenes* type 8 polysaccharide. Biochem: *9:* 2180–2185, 1970.
34. *Thurow, H., Choy, Y.-M., Frank, N., Niemann, H. & Stirm, S.:* Structure of *Klebsiella* serotype 11 capsular polysaccharide. Carbohyd. Res. *41:* 241–255, 1975.

Acta path. microbiol. scand. Sect. C, 83: 406–412, 1975

PRESENCE OF IMMUNOGLOBULIN-CONTAINING PLASMA CELLS IN TONSILS FROM TONSILLECTOMIZED CHILDREN

POUL AABEL ØSTERGAARD

Otological Department and Pathological Institute, Aalborg Sygehus Nord, DK-9000 Aalborg, Denmark

Østergaard, P. Aabel. Presence of immunoglobulin-containing plasma cells in tonsils from tonsillectomized children. Acta path. microbiol. scand. Sect. C, 83: 406–412, 1975.

Immunofluorescence investigations of IgG, IgA, IgM and IgE plasma cells in tonsil biopsies from 52 children, most of whom were suffering from chronic tonsillitis and frequent acute exacerbations of tonsillitis, are presented. The most prominent finding was a lack of IgE fluorescing plasma cells in 60 per cent of patients. IgA plasma cells were absent in 30 per cent of the patients and in 25 per cent IgA as well as IgE plasma cells were absent.

Key words: Immunoglobulin-containing plasma cells; tonsils; tonsillectomy.

Poul Aabel Østergaard, Department of Pediatrics, Odense Sygehus, DK-5000 Odense, Denmark.

Received 26.ii.75 Accepted 11.vi.75

IgA deficiency is supposed to be the most common immune defect (*Johansson et al.* 1968 b). Recent publications also postulate a rather frequent association between chronic upper respiratory infections (*Buckley et al.* 1968, *Bardare et al.* 1971), auto-immune diseases (*Ammann & Hong* 1970) and malignant, lymphoproliferative diseases (*Kersey et al.* 1974) among persons with isolated IgA deficiency.

IgE was isolated as a separate class of immunoglobulin in the late Sixties (*Ishizaka et al.* 1966, *Johansson et al.* 1968 a) and soon gained importance in the diagnosis of atopic diseases (*Johansson* 1967, *Wood & Oliver* 1972). Furthermore, some investigators have suggested that IgE, as well as IgA, play a role in the local immune defence of sinopulmonary mucous membranes (*Ammann et al.* 1969, *Cain et al.* 1969, *Ammann et al.* 1970).

The aim of the present study was to investigate whether defects in the immunoglobulin formation in children undergoing tonsillectomy could be demonstrated. For that purpose, tonsil tissue from 52 children, most of them with chronic tonsillitis and frequent attacks of acute tonsillitis, were examined for the presence of plasma cells containing IgG, IgA, IgM and IgE.

MATERIALS AND METHODS

The series comprises 52 children, four to fifteen years old, who were tonsillectomized during the period September to December 1973 at the Otological Department, Aalborg Hospital North. Tonsillectomy was performed on 38 of the patients because of frequently occurring tonsillitis and fever within two to four years, and on ten because of severe hypertrophy of the tonsils and consequent, partial obstruction of the pharynx, but absence of

a history of recurring tonsillitis. Tonsillectomy on the remaining 4 patients was indicated by atrophic tonsils and frequent attacks of tonsillitis. Three of the patients had a history of atopic diseases. The distribution of males and females was identical in the younger age group (four to nine years of age), whereas girls were more numerous in the older age group (ten to fifteen years of age) (Table 4).

Tissue preparation. Immediately after operation the tonsil in its entirety was wrapped in filter-paper moistened with 0.9 per cent saline and stored at a maximum for one hour at 4° C. Tissue for light and fluorescence microscopy was then cut from the tonsil pole approximately in the size of 5 × 5 mm, frozen immediately at – 70° C in 15 per cent gelatine without preservative and stored at that temperature until further preparation.

The rabbit-anti-human-IgG, -IgA, -IgM and -IgE used were purchased from Behringwerke AG, Germany. The batch numbers used and their molar Fluorescin/Protein (F/P) molar ratios and protein concentrations were as follows: IgG, batch numbers 564 A and 503 L (F/P molar ratio 0.6 and 1.0, protein concentration 3.1 and 3.5 g per cent respectively). IgA, batch numbers 504 F and 504 E (F/P molar ratio 1.3 and 1.3, protein concentration 3.4 and 3.4 g per cent respectively). IgM, batch numbers 595 A and 440 N (F/P molar ratio 0.5 and 0.3, protein concentration 4.0 and 3.5 g per cent respectively). IgE, batch numbers 514 D and 491 A (F/P molar ratio 0.9 and 1.1, protein concentration 3.4 and 3.4 g per cent respectively). The fluorescin labelled antisera were used in dilution 1:1, diluted in distilled water.

Toluidine blue and methyl pyronine green stained preparations. Before the examination of the preparations with FITC labelled antisera, a toluidine blue preparation was made from the tissue to secure that the tissue was cut from epithelial and subepithelial areas containing cells morphologically resembling plasma cells. As regards sections from the unfixed material to be used for immunofluorescent studies, the tissue was fixed in 10 per cent formaldehyde, and preparations stained with methyl pyronine green were made with a view to a final identification of plasma cells. The morphological criteria used were mononuclear cells of a spherical or often flattened form, a homogeneous strongly basophile cytoplasm, and a small, round or slightly oval, nucleus in an eccentric position.

Immunofluorescence examination. Cryostat sections of a thickness of 6 micron from the unfixed material were washed for ten minutes in Coon's buffer (pH 7.1) and incubated for 30 minutes at room temperature in a moist chamber together with the labelled antiserum. Excess of antiglobulin was removed by three washings in Coon's buffer, each lasting for ten minutes. Finally the sections were covered with clean coverslips using glycerin and buffer in the proportion 1:9 as adhesive. The preparations were examined by dark field fluorescence microscopy using a Leitz Orthoplan photomicroscope with an ultraviolet high pressure mercury lamp (HBO 200 w). The primary filter was a KP 490 mm interference filter with a red contrast band.

As a quantitative estimation cannot be performed by the employed single layer technique, a semiquantitative estimation was aimed at by cutting sections of dimensions of approximately 5×5 mm and subsequently counting all the fluorescing plasma cells in this area. The results are expressed as: — (no cells), + (—) (occasionally a single cell), + (2–4 cells), + + (5–10 cells), + + + (10–20 cells) and + + + + (more than 20 cells). Photographic recording of the preparations with bright fluorescence was made on high speed ektachrome colour film using a Leitz Orthomat camera with automatic exposure control. Times of exposure varied from 15 to 30 seconds. Performance of the investigations included always two preparations from each antiserum to be used as a control of the final result.

Blocking tests were carried out by exposing to (1) unlabelled rabbit-anti-human-IgG, -IgA, -IgM and -IgE and to (2) unlabelled rabbit-anti-human-albumin for 30 minutes. The sections were then washed with Coon's buffer for 3×5 minutes and incubated with FITC labelled antiserum for 15 minutes. Fluorescence was considered specific if the intensity of the fluorescence with labelled antisera was greater than that in the blocking tests using unlabelled antiglobulin (method 1) and unchanged in the blocking tests using anti-albumin (method 2). As control of autofluorescence, sections were incubated with Coon's buffer alone.

RESULTS

Toluidine blue and methyl pyronine green preparations constantly showed cells which morphologically resembled plasma cells. The plasma cells were usually accumulating in the subepithelial areas and in the periphery of the germinal centres, sometimes they may be infiltrating the tonsillar epithelium. The plasma cells were found in large numbers in hypertrophic tonsils, but were also invariably found in atrophic tonsils. The germinal centres were populated with small lymphocytes.

IgE fluorescing plasma cells were found in 21 (40 per cent) of the patients. However, in most of the remaining 60 per cent of the patients the germinal centres were found to be intensively stained with anti-IgE-FITC

a history of recurring tonsillitis. Tonsillectomy on the remaining 4 patients was indicated by atrophic tonsils and frequent attacks of tonsillitis. Three of the patients had a history of atopic diseases. The distribution of males and females was identical in the younger age group (four to nine years of age), whereas girls were more numerous in the older age group (ten to fifteen years of age) (Table 4).

Tissue preparation. Immediately after operation the tonsil in its entirety was wrapped in filter-paper moistened with 0.9 per cent saline and stored at a maximum for one hour at 4° C. Tissue for light and fluorescence microscopy was then cut from the tonsil pole approximately in the size of 5 × 5 mm, frozen immediately at – 70° C in 15 per cent gelatine without preservative and stored at that temperature until further preparation.

The rabbit-anti-human-IgG, -IgA, -IgM and -IgE used were purchased from Behringwerke AG, Germany. The batch numbers used and their molar Fluorescin/Protein (F/P) molar ratios and protein concentrations were as follows: IgG, batch numbers 564 A and 503 L (F/P molar ratio 0.6 and 1.0, protein concentration 3.1 and 3.5 g per cent respectively). IgA, batch numbers 504 F and 504 E (F/P molar ratio 1.3 and 1.3, protein concentration 3.4 and 3.4 g per cent respectively). IgM, batch numbers 595 A and 440 N (F/P molar ratio 0.5 and 0.3, protein concentration 4.0 and 3.5 g per cent respectively). IgE, batch numbers 514 D and 491 A (F/P molar ratio 0.9 and 1.1, protein concentration 3.4 and 3.4 g per cent respectively). The fluorescin labelled antisera were used in dilution 1:1, diluted in distilled water.

Toluidine blue and methyl pyronine green stained preparations. Before the examination of the preparations with FITC labelled antisera, a toluidine blue preparation was made from the tissue to secure that the tissue was cut from epithelial and subepithelial areas containing cells morphologically resembling plasma cells. As regards sections from the unfixed material to be used for immunofluorescent studies, the tissue was fixed in 10 per cent formaldehyde, and preparations stained with methyl pyronine green were made with a view to a final identification of plasma cells. The morphological criteria used were mononuclear cells of a spherical or often flattened form, a homogeneous strongly basophile cytoplasm, and a small, round or slightly oval, nucleus in an eccentric position.

Immunofluorescence examination. Cryostat sections of a thickness of 6 micron from the unfixed material were washed for ten minutes in Coon's buffer (pH 7.1) and incubated for 30 minutes at room temperature in a moist chamber together with the labelled antiserum. Excess of antiglobulin was removed by three washings in Coon's buffer, each lasting for ten minutes. Finally the sections were covered with clean coverslips using glycerin and buffer in the proportion 1:9 as adhesive. The preparations were examined by dark field fluorescence microscopy using a Leitz Orthoplan photomicroscope with an ultraviolet high pressure mercury lamp (HBO 200 w). The primary filter was a KP 490 mm interference filter with a red contrast band.

As a quantitative estimation cannot be performed by the employed single layer technique, a semiquantitative estimation was aimed at by cutting sections of dimensions of approximately 5×5 mm and subsequently counting all the fluorescing plasma cells in this area. The results are expressed as: — (no cells), + (—) (occasionally a single cell), + (2–4 cells), + + (5–10 cells), + + + (10–20 cells) and + + + + (more than 20 cells). Photographic recording of the preparations with bright fluorescence was made on high speed ektachrome colour film using a Leitz Orthomat camera with automatic exposure control. Times of exposure varied from 15 to 30 seconds. Performance of the investigations included always two preparations from each antiserum to be used as a control of the final result.

Blocking tests were carried out by exposing to (1) unlabelled rabbit-anti-human-IgG, -IgA, -IgM and -IgE and to (2) unlabelled rabbit-anti-human-albumin for 30 minutes. The sections were then washed with Coon's buffer for 3×5 minutes and incubated with FITC labelled antiserum for 15 minutes. Fluorescence was considered specific if the intensity of the fluorescence with labelled antisera was greater than that in the blocking tests using unlabelled antiglobulin (method 1) and unchanged in the blocking tests using anti-albumin (method 2). As control of autofluorescence, sections were incubated with Coon's buffer alone.

RESULTS

Toluidine blue and methyl pyronine green preparations constantly showed cells which morphologically resembled plasma cells. The plasma cells were usually accumulating in the subepithelial areas and in the periphery of the germinal centres, sometimes they may be infiltrating the tonsillar epithelium. The plasma cells were found in large numbers in hypertrophic tonsils, but were also invariably found in atrophic tonsils. The germinal centres were populated with small lymphocytes.

IgE fluorescing plasma cells were found in 21 (40 per cent) of the patients. However, in most of the remaining 60 per cent of the patients the germinal centres were found to be intensively stained with anti-IgE-FITC

TABLE 1. *Distribution of Ig-plasma Cell Fluorescence among Patients with Hypertrophic Tonsils and Recurrent Tonsillitis*

Patient no.	Sex	Age (years)	Immunofluorescent Studies			
			IgG	IgA	IgM	IgE
1	M	9	+ + +	—	+ (—)	+
2	M	9	+ + + +	+ + +	+ +	+ +
3	F	15	+ +	—	+	—
4	M	8	+ +	+ + +	+	+
5	M	13	+ (—)	+ +	+ (—)	+
6	F	15	+ +	+ +	+ +	—
7	F	11	+ +	+ +	+ +	+
8	M	6	+ + +	+ + +	+	—
9	F	13	+	+ +	+	+
10	F	12	+ +	+ + +	+ +	—
11	F	5	+ +	+ + +	+	+
12	M	6	+ +	—	+ +	—
13	F	7	+ +	+	+ + +	—
14	F	13	+ +	—	+	—
15	M	5	—	—	—	—
16	F	6	+	+	+	—
17	F	9	+ +	—	+ +	—
18	F	9	+ +	+ +	+	—
19	F	14	+	+ +	+	+ +
20	F	14	+ +	+ +	+	—
21	M	5	+	—	+	—
22	M	13	+ + +	+ +	+	—
23	F	13	+ +	+ (—)	+	+
24	F	8	+ + +	+ +	+	—
25	F	8	+ + + +	—	+	—
26	F	12	+ +	+ +	+	+
27	F	15	+ + +	+ +	+ +	+ +
28	F	12	+ +	+	+	—
29	M	6	+ +	+ +	+	—
30	F	13	+ +	—	+ +	+
31	F	14	+ +	+ +	+	+
32	M	7	+ + +	+ +	+	+
33	M	14	+ +	+	+	—
34	M	6	+ + +	—	+	—
35	M	4	+ +	+ +	+ +	—
36	F	6	+ +	+ +	+	+
37	M	5	+ +	+ +	+	+
38	F	12	+	—	+ + +	—

and seemed to be more hyperplastic than the preparations with positive IgE plasma cell fluorescence. A morphological *differentiation of the cells situated in the germinal centres* was not possible as the fluorescence was very homogeneous. Sections with Coon's buffer alone or sections from the blocking tests with unlabelled anti-immunoglobulin did not show the same germinal centre fluorescence, whereas sections with unlabelled anti-albumin from blocking test number 2 showed undiminished fluorescence.

IgA plasma cell fluorescence was observed in 36 (70 per cent) of the patients. In the cases without IgA fluorescing plasma cells (30 per cent), an immunofluorescence was also found as the *germinal centres* in these cases were *not stained* with the labelled anti-serum.

IgG and IgM fluorescing plasma cells were

TABLE 2. *Distribution of Ig-plasma Cell Fluorescence among Patients with Severe Hypertrophic Tonsils but without Recurrent Tonsillitis*

Patient no.	Sex	Age (years)	Immunofluorescent Studies			
			IgG	IgA	IgM	IgE
1	M	9	—	—	+	—
2	F	5	+ +	+ +	+	—
3	F	9	—	+ +	—	—
4	F	5	+	+ +	+	+ +
5	F	4	+	—	+	—
6	M	4	+ +	+	+	—
7	F	12	+ +	+ +	+	+
8	F	7	+ + +	—	+ +	+ +
9	M	4	+ + +	+ (—)	+	—
10	M	13	+ +	—	+ +	—

TABLE 3. *Distribution of Ig-plasma Cell Fluorescence among Patients with Atrophic Tonsils and Recurrent Tonsillitis*

Patient no.	Sex	Age (years)	Immunofluorescent Studies			
			IgG	IgA	IgM	IgE
1	F	11	+ +	+ + +	+	+
2	F	15	+ + +	+ +	+ +	—
3	F	15	+ +	+	+	—
4	F	14	+ + +	—	+	+ +

TABLE 4. *Absence of the Various Plasma Cells in Tonsillar Sections According to Sex and Age of Patients*

Age (years)	Sex	Total number	IgG	IgA	IgM	IgE	Combined IgA/IgE deficiency
4-9	M	15	2	6	1	9	5
4-9	F	13	1	5	1	10	4
10-15	M	4	0	1	0	3	1
10 15	F	20	0	4	0	9	3

demonstrated in 94 per cent and 96 per cent of the patients, respectively. In one patient (no. 15, Table 1) it was not possible to find fluorescing plasma cells at all, in spite of an unmistakable, although sparse, occurrence of plasma cells in the methyl pyronine green preparation.

Finally, a combined lack of IgA and IgE fluorescing plasma cells was observed in 13 patients (25 per cent), mainly among the younger patients (Table 2).

The lack of fluorescing plasma cells was most common in the younger age group, while the marked deficiency in IgE plasma cells appeared more regularly in the series as a whole (Table 4). The series does not permit conclusions concerning possible differences in the occurrence of the various Ig-containing plasma cells between (1) hypertrophic tonsils and a history of recurrent tonsillitis, (2) hypertrophic tonsils without frequent infections of the throats and (3) atrophic tonsils

and recurrent tonsillitis (Table 1, 2 and 3) since group 1 constitutes too big a part of the series.

IgG fluorescing plasma cells were found to be most numerous in these patients, most often between ++ and +++ (Tables 1, 2 and 3). Next in frequency come the IgM plasma cells, fluctuating between + and ++. The demonstration of IgA and IgE plasma cells was very inconstant and, if demonstrated, it would be fluctuating between ++ and +++ and + and ++, respectively.

DISCUSSION

The most prominent finding in this investigation was the lack of IgE fluorescing plasma cells in 60 per cent of the patients. In addition, the absence of IgA plasma cells in 30 per cent of the children, and a lack of IgA and IgE in 25 per cent, was unexpected. These data are not in agreement with the results obtained in earlier investigations. Thus, in investigations of human adenoids *Crabbé & Heremans* (1967) constantly demonstrated both IgG, IgA and IgM plasma cells. This was the case also in a study of human tonsils by *Tada & Ishizaka* (1970). However, the predominance of IgG plasma cells demonstrated in the present study was also observed by the authors mentioned. Concerning the number of IgE plasma cells in human adenoids and tonsils, *Tada & Ishizaka* (1970) estimated it to be ten times less frequent than the occurrence of IgG and IgA plasma cells.

The shortcomings of previous investigations as well as of the present study is the lack of a control material consisting of tonsillar tissue from children without a history of recurrent tonsillitis. The procurement of this type of control material involves an ethical as well as a legal dilemma (*Lowe et al.* 1974). Tonsils obtained at autopsies might be a possibility, but immunofluorescent investigations are very sensitive to the unavoidable time lag between time of death and removal of the tissue. Furthermore, if tonsils obtained at autopsies were to be used, the difficulties concerning anamnestic data of the controls would be considerable. In the absence of adequate controls, the severe IgA and IgE deficiency suggested by the present data should therefore be taken with some reservation.

The bright fluorescence of the germinal centres but absence of simultaneous plasma cell fluorescence in preparations with anti-IgE-FITC is very difficult to explain. On the assumption that it represents a specific reaction, it cannot be precluded that a certain maturation arrest between plasma cell precursors situated in the germinal centres and the fully developed IgE producing plasma cell occurs in some patients with chronic tonsillitis. It is worthy of note that levels of IgE in serum also were low in most of these patients (*Østergaard*). A similar hypothesis was advanced by *Schulte-Wissermann et al.* (1974) who reported on a boy in whom deficiency in serum IgG and IgA was observed. The patient concerned presented hyperplastic germinal centres but according to repeated lymph node biopsies, plasma cells were lacking.

In the present study the rather frequently observed absence of fluorescing IgA plasma cells was another striking feature and the same applies to absence of both IgA and IgE plasma cells in 30 per cent and 25 per cent of the patients, respectively, especially among the children in the younger age group (Table 4). These observations may only reflect that development of the IgA and IgE systems is delayed as compared with the IgG and IgM synthesis, but may also be an expression of a more fundamental difference between the two age groups with regard to the basic, immunological cause of the tendency to a development of chronic and recurrent tonsillitis in these cases. Yet, there was no relationship between the immunological findings and the pre-operative severity of symptoms (*Østergaard*).

The significance of IgA in the defence of mucous membranes against infections has been documented repeatedly (*South et al.* 1968, *Bellanti* 1971), whereas the role of IgE in this connection still is obscure. Several investigators have found a severe deficiency in

serum IgA among patients with recurrent upper respiratory infections (*Buckley et al.* 1968, *Buser et al.* 1968, *Bardare et al.* 1971). There are also some indications of a probable connection between combined IgA and IgE deficiency and recurrent sinopulmonary infections, especially among patients with ataxia-telangiectasia (*South et al.* 1968, *Ammann et al.* 1969), but so far, the mechanism behind is unknown. Combined IgA/IgE deficiency has also been observed in a patient without ataxia-telangiectasia (*Ammann et al.* 1970) and in a report by *Cain et al.* (1969) a patient with chronic sinopulmonary infections and isolated IgE deficiency in serum was discussed.

Veltri et al. (1972) demonstrated normal serum- and secretory IgA levels in 17 children on whom tonsillectomy was performed. On the other hand, *Donovan & Soothill* (1973) found the IgA level in serum to be low in 36 children on whom tonsillectomy was performed because of recurrent sore throats. They also found weak or no lymphocyte response to phytohaemagglutinin in some of the patients with low IgA. *Donovan & Soothill* estimated also the IgE in serum in eleven patients and found that this immunoglobulin level was normal. The authors postulated that children with recurrent sore throat represented a group of individuals in whom "minor immunodeficiency" was present.

It is possible that the lack of IgA and/or IgE fluorescing plasma cells in the patients comprised in this series is the primary cause of chronic infection involving frequent, acute attacks of tonsillitis. If true, every tendency to disturb the mutual balance between the two immunoglobulins may tend to increase the frequency of respiratory infections.

The investigations were in part supported by grants from *Det Lægevidenskabelige Forskningsfond For Nordjyllands Amt*. The author is grateful to Dr. *J. W. Paulsen*, Department of Otolaryngology and to Dr. *H. Olesen*, Institute of Pathology, Aalborg Hospital North, for excellent working conditions. The author is also indepted to Dr. *H. J. Andersen*, Department of Pediatrics and to Professor *S.-E. Svehag*, Institute of Microbiology, Odense University Hospital, for critical evaluation of the paper. A special thank to Miss *Esther Schwartz* for skilful technical assistance.

REFERENCES

Ammann, A. J., Cain, W. A., Ishizaka, K., Hong, R. & Good, R. A.: Immunoglobulin E deficiency in ataxia-telangiectasia. New Engl. J. Med. *281*: 469–472, 1969.

Ammann, A. J. & Hong, R.: Selective IgA deficiency and autoimmunity. Clin. exp. Immunol. *7*: 833–838, 1970.

Ammann, A. J., Roth, J. & Hong, R.: Recurrent sinopulmonary infections, mental retardation, and combined IgA and IgE deficiency. J. Pediat. *77*: 802–804, 1970.

Bardare, M., Cislaghi, G. W. & Zuccoli, G.: Possible relationships between IgA deficiency and recurrent sinopulmonary infections. Helv. Paediat Acta *2*: 173–178, 1971.

Bellanti, J. A.: Biologic significance of the secretory gamma A globulins. Pediatrics *48*: 715–729, 1971.

Buckley, R. H., Dees, S. C. & O'Fallon, W. M.: Serum immunoglobulins. II. Levels in children subject to recurrent infections. Pediatrics *84*: 50–60, 1968.

Buser, F., Butler, R. & Martin DU Paw, R.: Susceptibility to infections and IgA deficiency in the infant. J. Pediat. *72*: 29–35, 1968.

Cain, W. A., Ammann, A. J., Hong, R., Ishizaka, K. & Good, R. A.: IgE deficiency associated with chronic sinopulmonary infection. J. Clin. Invest. *48*: 12a–13a, 1969.

Crabbe', P. A. & Heremans, J. F.: Distribution in human nasopharyngeal tonsils of plasma cells containing different types of immunoglobulin polypeptide chains. Lab. Invest. *16*: 112–123, 1967.

Donovan, R. & Soothill, J. F.: Immunological studies in children undergoing tonsillectomy. Clin. exp. Immunol. *14*: 347–357, 1973.

Ishizaka, K., Ishizaka, T. & Hornbrook, M. M.: Physio-chemical properties of human reaginic antibody. J. Immunol. *97*: 75–85, 1966.

Johansson, S. G. O.: Raised level of a new immunoglobulin class (IgND) in asthma. Lancet *ii*: 951–953, 1967.

Johansson, S. G. O., Bennich, H. & Wide, L.: A new class of immunoglobulin in human serum Immunology *14*: 265–272, 1968 a.

Johansson, S. G. O., Högman, E. F. & Killander, J.: Quantitative immunoglobulin determinations. Acta path. microbiol scand. *74*: 519–524, 1968 b.

Kersey, J. H., Spector, B. D. & Good, R. A.: Cancer in children with primary immuno-deficiency diseases. J. Pediat. *84*: 263–264, 1974.

Lowe, C. U., Alexander, D. & Mishkin, B.: Nontherapeutic research in children: An ethical dilemma. J. Pediat. *84:* 468–472, 1974.
Schulte-Wissermann, H., Lemmel, E. M., Opferkuch, W., Grosse-Wilde, H., Mempel, W., Kössling, F. K., & Cottier, H.: Immunmangelsyndrom mit dysgammaglobulinämie und lymphatischer hyperplasie. Helv. Paediat. Acta *28:* 569–581, 1974.
South, M. A., Cooper, M. D., Wollheim, F. A. & Good, R. A.: The IgA system. The clinical significance of IgA deficiency: Studies in patients with agammaglobulinemia and ataxia-telangiectasia. Am. J. Med. *44:* 168–178, 1968.
Tada, T. & Ishizaka, K.: Distribution of IgE forming cells in lymphoid tissues of the human and monkey. J. Immunol. *104:* 377–387, 1970.
Veltri, R. W., Sprinkle, P. M., Keller, S. A. & Chicklo, J. M.: Immunoglobulin changes in a pediatric otolaryngic sample subsequent to T & A. J. Laryng. Otol. *86:* 905–916, 1972.
Wood, C. B. S. & Oliver, J.: Serum IgE in asthmatic children. Arch. Dis. Childh. *47:* 890–896, 1972.
Østergaard, P. Aa. (unpublished results).

Acta path. microbiol. scand. Sect. C, *83:* 413–422, 1975

REJECTION PATTERNS OF THREE HUMAN MALIGNANT TUMOURS TRANSPLANTED IN THYMUS GRAFTED NUDE MICE

C. O. Povlsen and J. Rygaard

Pathological Anatomical Institute, Kommunehospitalet, The University Institute of Pathological Anatomy, and Pathological Anatomical Institute, Gentofte Amtssygehus, Copenhagen, Denmark

Povlsen, C. O. & Rygaard, J. Rejection patterns of three human malignant tumours transplanted in thymus grafted nude mice. Acta path. microbiol. scand. Sect. C, *83:* 413–422, 1975.

Nude mice suffering from congenital thymic aplasia will accept human malignant tumours. This study concerns the effect of grafting of neonatal thymus in nude mice subsequently transplanted with human malignant tumours serially grown in nude mice. Three different human tumours —a malignant melanoma, a Burkitt's lymphoma and an epidermoid carcinoma—were rejected by nude mice supposed to be fully immunologically competent following thymus grafting. The rejection process in its various stages was studied by transplanting the malignant melanoma to nude mice, still in the process of immunological reconstitution. There was a considerable variation in the development of immunological competence following thymus grafting. Rejection time varied from 14 to 132 days after thymus transplantation. The rejection process differed microscopically from a classical cell-mediated immune response in a striking paucity of lymphocytes. A histological grading of the time course of the rejection process is set forth and discussed

Key words: Heterotransplantation; thymus transplantation; human malignant tumour; nude mice, graft rejection.

C O Povlsen, The University Institute of Pathological Anatomy, Juliane Mariesvej 16, DK-2100 Copenhagen, Denmark.

Received 7.vii.75 Accepted 7.viii.75

Nude mice, suffering from congenital thymic aplasia (*Pantelouris* 1968), will accept transplants of allogeneic and heterogeneic tissue—both normal and tumour tissue (*Rygaard* 1969, *Rygaard & Povlsen* 1969, *Povlsen & Rygaard* 1971, 1972).

It has previously been shown that transplantation of neonatal thymus in nude mice will reconstitute their capability to reject transplants of foreign skin (*Rygaard* 1973).

The defence mechanisms of the organism against malignant tumours are of particular interest.

The effect of thymus grafting in nude mice, subsequently transplanted with human malignant tumours has not previously been investigated. The purpose of the present study is to elucidate the results of transplantation of three different human malignant tumours in nude mice, supposed to be fully immunologically competent following thymus graft-

Lowe, C. U., Alexander, D. & Mishkin, B.: Non-therapeutic research in children: An ethical dilemma. J. Pediat. *84:* 468–472, 1974.

Schulte-Wissermann, H., Lemmel, E. M., Opferkuch, W., Grosse-Wilde, H., Mempel, W., Kössling, F. K., & Cottier, H.: Immunmangelsyndrom mit dysgammaglobulinämie und lymphatischer hyperplasie. Helv. Paediat. Acta *28:* 569–581, 1974.

South, M. A., Cooper, M. D., Wollheim, F. A. & Good, R. A.: The IgA system. The clinical significance of IgA deficiency: Studies in patients with agammaglobulinemia and ataxia-telangiectasia. Am. J. Med. *44:* 168–178, 1968.

Tada, T. & Ishizaka, K.: Distribution of IgE forming cells in lymphoid tissues of the human and monkey. J. Immunol. *104:* 377–387, 1970.

Veltri, R. W., Sprinkle, P. M., Keller, S. A. & Chicklo, J. M.: Immunoglobulin changes in a pediatric otolaryngic sample subsequent to T & A. J. Laryng. Otol. *86:* 905–916, 1972.

Wood, C. B. S. & Oliver, J.: Serum IgE in asthmatic children. Arch. Dis. Childh. *47:* 890–896, 1972.

Østergaard, P. Aa. (unpublished results).

TABLE 1. *Origin, Histology and Transfer Number of Three Human Malignant Tumours Serially Grown in Nude Mice*

Patient data Age	Sex	Record no.	Origin of tumour material	Type of tumour	Transfer no.	Ref.
60	♀	P 2800	Subcutaneous metastasis of right femur from malignant melanoma of right lower leg.	Amelanotic malignant melanoma.	22–25	*Visfeldt et al.* 1972
7	♀	IP 14858	Biopsy from tumour involving both ovaries and kidneys.	Burkitt's lymphoma.	19	*Povlsen et al.* 1973
84	♀	KH 892/70	Lymphnode metastasis of right ingven from primary tumour of vulva.	Well differentiated epidermoid carcinoma with cornification.	20	*Povlsen*, to be published

TABLE 2. *Fate of Three Human Malignant Tumours Heterotransplanted in Thymus Grafted Nude Mice 40–50 Days Following Thymus Grafting*

Tumour	Number of mice	Interval thymus grafting-tumour grafting days	Period of observation following grafting of thymus days	tumour days	Fate of tumour No take	Reject	Accept	Reconstituted of total
Malignant melanoma	6	40–41	64–88	24–47	5		1	5/6
	3	controls		47			3	0/3
Burkitt's lymphoma	8	47–48	98–99	51	8			8/8
	4	controls		31–51			4	0/4
Epidermoid carcinoma	7	45–50	142–147	97	4	3*)		7/7
	4	controls		11–36			4	0/4

*) Tumour growth curve reversal days 34, 40 and 82 following tumour implantation, i.e. days 79, 85 and 132 following thymus grafting.

bodies of the spleen (*Parrott et al* 1966, *de Sousa et al.* 1969, *Rygaard & Povlsen* 1974 b).

3) Lymphocyte counts exceeding 1400/microliter (*Rygaard & Povlsen* 1974 b).

In our first series, 20 out of 21 thymus grafted nude mice were reconstituted, whereas none of 11 controls were so. The distribution of reconstituted animals is shown in Table 2. In our second series 31 of 32 thymus grafted nude mice, and none of 21 controls were reconstituted (Table 3).

Fate of Three Human Malignant Tumours Inoculated in Control Animals and Nude Mice 40–50 Days Following Thymus Grafting (Table 2)

Control mice. Tumour transplants were accepted in all 11 control mice. Growth patterns of tumours were as described else-

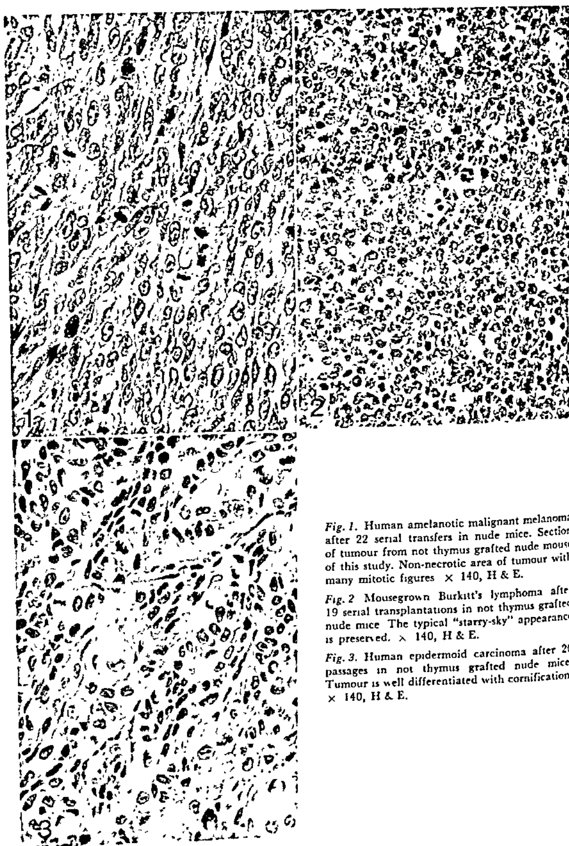


Fig. 1. Human amelanotic malignant melanoma after 22 serial transfers in nude mice. Section of tumour from not thymus grafted nude mouse of this study. Non-necrotic area of tumour with many mitotic figures × 140, H & E.

Fig. 2 Mousegrown Burkitt's lymphoma after 19 serial transplantations in not thymus grafted nude mice The typical "starry-sky" appearance is preserved. × 140, H & E.

Fig. 3. Human epidermoid carcinoma after 20 passages in not thymus grafted nude mice. Tumour is well differentiated with cornification. × 140, H & E.

TABLE 3. *Fate of Human Malignant Melanoma Heterotransplanted in Thymus Grafted Nude Mice 14–17 Days Following Thymus Grafting*

Number of mice	Interval thymus grafting-tumour grafting days	Period of observation following grafting of thymus	tumour (days)	Fate of tumour No take	Reject	Accept	Reconstituted of total
32	14–17	40–48	26–31	8	18*)	6	31/32
21	controls		29–31			21	0/21

*) Tumour growth curve reversal interval: days 10–25 following tumour implantation, i.e. days 24–39 following thymus grafting.

where (*Povlsen* & *Jacobsen* 1975, *Povlsen* & *Rygaard* 1974, *Povlsen*, to be published).

The microscopical examination of tumours from control animals showed in all cases a histological appearance in full accordance with the human donor material as described elsewhere (*Visfeldt et al.* 1972, *Povlsen et al.* 1973, *Povlsen*, to be published) and as shown in Figs. 1–3. In tumours measuring more than 5 mm in diameter, central necrosis was found with slight infiltration of polymorphnuclear neutrophil granulocytes. In tumour stroma few or no granulocytes and a few mononuclear cells were seen, but otherwise no signs of host reaction, cfr. Figs. 1–3.

Thymus grafted nude mice. In 20 out of 21 thymus grafted nude mice, tumour transplants were not accepted or were rejected at varying intervals following thymus transplantation. Details concerning the three test tumours are given below.

Malignant melanoma: the tumour graft was not accepted in 5 out of 6 animals. These 5 animals were all reconstituted. There appeared at the site on inoculation—after a latency period of seven days—a yellow, soft swelling, maximally measuring $5 \times 5 \times 6$ mm. Thereafter, the local swelling receded within two weeks. Histological examination of tissue from the inoculation site showed sparse fibrous connective tissue infiltrated with a few polymorphnuclear neutrophil granulocytes and a few macrophages, but no tumour tissue (Fig 4). In one animal the tumour was accepted, reaching a maximal size of $8 \times 8 \times 6$ mm by day 46 following thymus grafting, at which time the animal died spontaneously. This animal showed no signs of reconstitution. Histologically the tumour could not be distinguished from tumours in not thymus grafted nude mice.

Burkitt's lymphoma: tumour transplants of Burkitt's lymphoma were not accepted in 8 out of 8 thymus grafted nude mice. The course of rejection was as described above. All animals were reconstituted.

Epidermoid carcinoma: transplants of epidermoid carcinoma were not accepted in 4 out of 7 thymus grafted recipients. The microscopical examination of the inoculation site revealed sparse keratin with small islands of tumour tissue, all showing total coagulation necrosis. Dense connective tissue, infiltrated with macrophages surrounded the necrotic areas (Fig. 5). Three tumours were rejected In these animals local tumour growth was observed after a latency period of one week. Tumour growth was as in not thymus grafted controls until the time of rejection. The time of rejection has been defined as the time of reversal of the tumour growth curve. There is considerable variation between animals, with reversal times between day 79, 84, and 132 following thymus grafting. At the same time the macroscopical appearance of tumours changed. Within one week, the firm, well defined process, not differing in colour from the surrounding skin, changed into an ill defined, soft, yellow swelling. Simultaneously the skin over the tumour turned red. In one case, there was ulceration of the skin with expulsion of yellow



Fig. 4. Section from site of implantation of nude mouse transplanted with human malignant melanoma. In the subcutaneous space is sparse connective tissue infiltrated with macrophages and polymorphnuclear granulocytes, but no tumour tissue. Day 88 after thymus grafting. Day 74 after tumour inoculation. × 60, H & E.

Fig. 5. Section from site of implantation of nude mouse transplanted with human epidermoid carcinoma. Remnants of keratin surrounded by dense connective tissue. Day 142 after thymus grafting. Day 97 after tumour transplantation. × 60, H & E.

necrotic material and subsequent healing of the skin. In two other instances tumours were totally resorbed within two weeks. By histological examination of the inoculation site no keratin or tumour remnants were found in the 3 animals, but exclusively connective tissue infiltrated with macrophages. All 7 animals were reconstituted.

Fate of Malignant Melanoma in Control Mice and Nude Mice 14–17 Days Following Thymus Transplantation

Control Mice. In all 21 control animals the tumour transplant was accepted. Growth pattern and histological appearance were as described above and as shown in Fig. 1.

Thymus grafted nude mice. Tumour transplant was not accepted in 8 animals. The macroscopical and microscopical appearance corresponded with the description given for malignant melanoma in our first series. In 18 animals tumours were rejected between day 24 and 39 following thymus grafting. The histological examination of these tumours showed much variation. Changes are described below according to their severity.

Slight changes (Fig. 6): no changes in tumour parenchyma. Stroma and peritumoural tissue: increased cellularity, predominantly polymorphnuclear neutrophil granulocytes, eosinophil granulocytes and macrophages, and a few lymphocytes

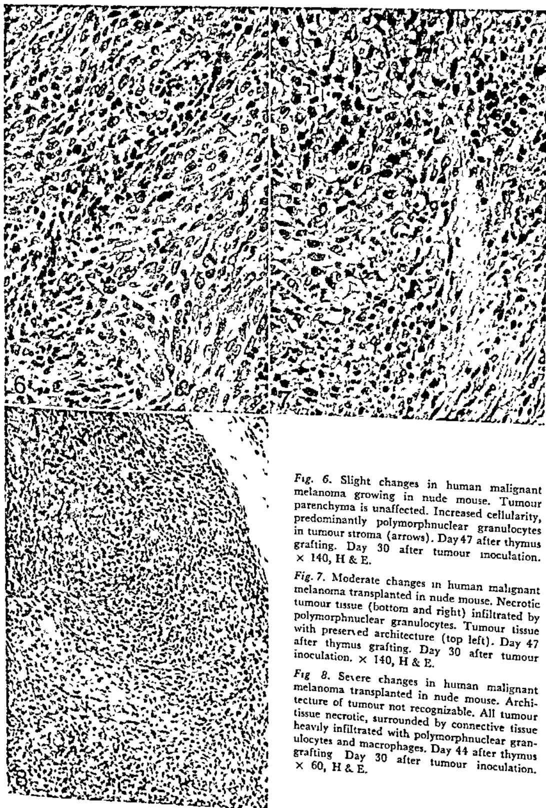


Fig. 6. Slight changes in human malignant melanoma growing in nude mouse. Tumour parenchyma is unaffected. Increased cellularity, predominantly polymorphnuclear granulocytes in tumour stroma (arrows). Day 47 after thymus grafting. Day 30 after tumour inoculation. × 140, H & E.

Fig. 7. Moderate changes in human malignant melanoma transplanted in nude mouse. Necrotic tumour tissue (bottom and right) infiltrated by polymorphnuclear granulocytes. Tumour tissue with preserved architecture (top left). Day 47 after thymus grafting. Day 30 after tumour inoculation. × 140, H & E.

Fig 8. Severe changes in human malignant melanoma transplanted in nude mouse. Architecture of tumour not recognizable. All tumour tissue necrotic, surrounded by connective tissue heavily infiltrated with polymorphnuclear granulocytes and macrophages. Day 44 after thymus grafting Day 30 after tumour inoculation. × 60, H & E.

Moderate changes (Fig. 7): tumour parenchyma: coagulation necrosis in some areas, whereas others are unaffected. Stroma and peritumoural tissue: extension of changes described above with proliferation of vessels and fibroblasts in all septa, including central areas of tumour. Heavy cellular infiltration as described above, still with relatively few lymphocytes.

Severe changes (Fig. 8): all tumour tissue necrotic, but not fully resorbed. The architecture of tumour tissue is hardly or not discernible. In the terminal stage, tumour tissue has disappeared completely, and only fibrous connective tissue with few macrophages is found at the inoculation site, cfr. Fig. 4.

Six tumours of this series still grew at the termination of the experiment. Microscopically these tumours corresponded to tumours in not thymus grafted nude mice. One of these animals was not reconstituted, whereas all other animals of the series were so.

DISCUSSION

The study has shown a deleterious effect of a functioning thymus graft on the three usually readily accepted and steadily growing tumours. Our studies of intermediate stages in the rejection process have revealed a considerable variation among animals, and have allowed a histological grading of the course of rejection.

Reconstitution

The criteria for selection of supposedly reconstituted animals—weight gain and good general condition—seemed to be suitable, in that 51 out of 53 thymus transplanted nude mice thus selected eventually appeared to be reconstituted This final judgment was based on several parametres, but it is noteworthy that rapid weight gain within the experimental period, so easily observed, would in itself be a sufficient criterium in this material. The critical lymphocyte count—1400 per microliter—was chosen on the basis of our previous studies. By analysis of variance we found this characteristic effect of homozygosity of the *nu*-gene in nude mice of three genetic backgrounds *versus* normal mice of the same inbred strains (*Rygaard & Povlsen* 1974 b). The present study of not thymus grafted nude mice has confirmed this finding. Lymphocyte counts of nude mice are normalized following thymus grafting. This would indicate that the lymphopenia of nude mice must be secondary to the absence of a thymus, rather than a primary effect of the gene. The histological appearance of lymph nodes, Peyer's patches, and spleen at the time of observation chosen (6 weeks or more following thymus grafting) is uniform. This finding corresponds to previous investigations by *Rygaard* (1973), and to *de Sousa et al.*'s (1974) investigations of lymphoid tissue in thymus grafted nude mice.

Transplantation of Three Different Malignant Tumours in Nude Mice 40–50 Days Following Thymus Grafting

All reconstituted animals rejected their tumours. All transplants of malignant melanoma and Burkitt's lymphoma, and 4 out of the 7 epidermoid carcinoma transplants listed in Table 2 as "no take" must be considered rejected within 8 days, when compared with the 100 per cent take of tumours in control animals. Three out of 7 animals inoculated with epidermoid carcinoma did not reject their tumours until day 34, 40 and 82 following implantation, coresponding to day 79, 85, and 132 following thymus grafting. The reasons for these discrepancies are obscure. Possible explanations include differences in tumour antigenecity and variation in development of immunological competence at the time of tumour transplantation. Histological examination of the inoculation site in animals transplanted with malignant melanoma and Burkitt's lymphoma showed, as could be expected at this time, only scar tissue. The presence of remnants of keratin in animals inoculated with epidermoid carcinoma may be due to the resistance of keratin to lysis and phagocytosis.

Transplantation of Malignant Melanoma in Nude Mice 14–17 Days Following Thymus Grafting

Whereas the tumour transplantation so far discussed represent a *fait accompli* at the time of observation, the purpose of our second series was to elucidate the tumour rejection process in its various stages and at the same time get an impression of the developing immunological competence, leading to rejection of the tumour graft. The test tumour of this series was chosen because of its high percentage of take and the rapid growth rate in nude mice. The interval between thymus grafting and tumour transplantation turned out to be well chosen. Only 8 out of 32 animals were so reconstituted at the time of tumour transplantation that the transplant did not take. Eighteen animals showed varying stages of rejection, and thus allowed the observations which were the object of this part of our study. Finally, tumour grafts were still accepted in 6 animals, one of which was not reconstituted The variation in the degree of reconstitution among animals is considerable, but is in accordance with the findings in our first series as far as concerns the epidermoid carcinoma, although with some displacement in time.

The course of rejection in the 18 animals, rejecting already established tumours within the observation period, is of particular interest. The time of rejection of tumours within this group varies between 10 and 25 days following tumour inoculation, corresponding to 24 to 39 days following thymus grafting. There is thus a gradual transition both to the group not accepting their tumours at all (i.e. rejecting within the first week) and to the group still accepting their tumours at the termination of experiment. On the basis of results in our first series, one may presume that all tumours would have been rejected within further one to two weeks.

Loor & Kindred (1974) described a considerable variation in the degree of immunological competence among nude mice following thymus grafting. However, the variation found in our study, from day 14 to day 132. while confirming their finding, extends it to an astonishing degree.

Histological Examination of the Rejection Process

Our studies of the rejection of already established tumours, showing no signs of host reaction, have facilitated observation of the rejection process from the very beginning, not blurred by or mixed up with healing processes as would be the situation in studies of e.g. incompatible grafts in already immunologically competent recipients. In "Results" we classified the histological changes according to their severity, and we feel that this classification can without difficulty be interpreted as the time course of the process. The rejection process in thymus grafted nude mice differs from a classical, cell-mediated immune response/graft rejection in respect to the striking paucity of lymphocytes and plasma cells. This inflammatory response is totally nonspecific and corresponds to the reaction to e.g. necrosis. If tumour necrosis caused the inflammatory response, the question arises whether the necrosis was induced by a cell-mediated or humoral immune response. Our results do not answer this question, but further studies are in progress.

Tumour transplanted nude mice seem to be a suitable model in the study of developing immunological competence.

The study has shown that thymus grafted nude mice will reject the three human malignant tumours at a time when full immunological competence has been achieved. Also tumours implanted in nude mice still in the process of reconstitution will be recognized as "not self". The course of events if tumour is transplanted prior to thymus—whether it will be tolerated as "self" by the neonatal thymus graft or rejected—is under investigation.

The authors acknowledge the expert technical assistance of Miss *Margit Bæksted* and Mr. *Oluf Rasmussen*. This work was supported by grants from *The Danish Medical Research Council* and *The Danish Cancer Society*.

REFERENCES

1. *Loor, F. & Kindred, B.:* Immunofluorescence study of T cell differentiation in allogeneic thymus-grafted nudes. In: Rygaard, J. & Povlsen, C. O. (Eds.): Proceedings of the first international workshop on nude mice. Gustav Fischer Verlag, Stuttgart 1974. pp. 141–147.
2. *Pantelouris, E. M.:* Absence of thymus in a mouse mutant. Nature *217:* 370–371, 1968.
3. *Parrott, D. M. V., de Sousa, M. A. B. & East, J.:* Thymus-dependent areas in the lymphoid organs of neonatally thymectomized mice. J. Exp. Med. *123:* 191–203, 1966.
4. *Povlsen, C. O., Fialkow, P. J., Klein, E., Klein, G., Rygaard, J. & Wiener, F.:* Growth and antigenic properties of a biopsy-derived Burkitt's lymphoma in thymus-less (nude) mice. Int. J. Cancer *11:* 30–39, 1973.
5. *Povlsen, C. O. & Jacobsen, G. K.:* Chemotherapy of human malignant melanoma transplanted in nude mice. Cancer Res. (in press).
6. *Povlsen, C. O. & Rygaard, J.:* Heterotransplantation of human adenocarcinomas of the colon and rectum to the mouse mutant nude. A study of nine consecutive transplantations. Acta path. microbiol. scand. Sect. A, *79:* 159–169, 1971.
7. *Povlsen, C. O. & Rygaard, J.:* Heterotransplantation of human epidermoid carcinomas to the mouse mutant nude. Acta path. microbiol. scand. Sect. A, *80:* 713–717, 1972.
8. *Povlsen, C. O. & Rygaard, J.:* Effects of *cyclophosphamide (Endoxan®) on a Burkitt's* lymphoma serially grown in nude mice. In: Rygaard, J. & Povlsen, C. O. (Eds.): Proceedings of the first international workshop on nude mice. Gustav Fischer Verlag, Stuttgart 1974. pp. 285–292.
9. *Rygaard, J.:* Immunobiology of the mouse mutant "nude". Preliminary Investigations. Acta path. microbiol. scand. *77:* 761–762, 1969.
10. *Rygaard, J.:* Thymus & Self. Immunobiology of the mouse mutant nude. F.A.D.L., Copenhagen, and John Wiley & Sons, London, N.Y., Sydney, 1973. pp. 50–59 & pp. 124–136.
11. *Rygaard, J. & Povlsen, C. O.:* Heterotransplantation of a human malignant tumour to "nude" mice. Acta path. microbiol. scand. *77:* 758–760, 1969.
12. *Rygaard, J. & Povlsen, C. O.:* The mouse mutant nude does not develop spontaneous tumours. Acta path. microbiol. scand. Sect. B, *82:* 99–106, 1974 a.
13. *Rygaard, J. & Povlsen, C. O.:* Effects of homozygosity of the nude (nu) *gene in three* inbred strains of mice. Acta path. microbiol. scand. Sect. A, *82:* 48–70, 1974 b.
14. *de Sousa, M. A. B., Parrott, D. M. V. & Pantelouris, E. M.:* The lymphoid tissue in mice with congenital *aplasia of the* thymus. Clin. exp. Immunol. *4:* 637–644, 1969.
15. *de Sousa, M. A. B., Pritchard, H. & Parrott, D. M. V.:* An analysis of some morphological features of the lymphoid system in nude mice. In: Rygaard, J. & Povlsen, C. O. (Eds.): Proceedings of the first international workshop on nude mice. Gustav Fischer Verlag, Stuttgart 1974. pp. 119–126
16. *Visfeldt, J., Povlsen, C. O. & Rygaard, J.:* *Chromosome* analysis of human tumours following heterotransplantation to the mouse mutant nude. Acta path. *microbiol.* scand. Sect. A, *80:* 169–176, 1972

Acta path. microbiol. scand. Sect. C, *83:* 423–428, 1975

ANTIBODIES TO *ASPERGILLUS FUMIGATUS*. CHARACTERIZATION OF A HAEMAGGLUTINOGEN

MARIANNE RÖDSÆTHER and O. TÖNDER

The Gade Institute, Department of Microbiology and Broegelmann Research Laboratory for Microbiology, School of Medicine, The University of Bergen, Bergen, Norway

Rödsæther, Marianne & Tönder, O. Antibodies to *Aspergillus fumigatus*. Characterization of a haemagglutinogen. Acta path. microbiol. scand. Sect. C, *8:* 423–428, 1975.

Rabbit antiserum to *A. fumigatus* and sera from patients with and without aspergillosis were tested for anti-haemolysins, CFT antibodies, precipitins and haemagglutinins using extracts of homogenized, disrupted fungi as antigen preparations. The rabbit serum reacted in all 4 tests, while the human sera gave significant reactions in precipitation and tanned cell indirect haemagglutination (IHA) only. Of 9 human sera which gave precipitation in double diffusion in agar, only the 5 which were positive in IHA were from patients with definite aspergillosis. Some sera precipitated extracts of fungi of other species, and even of other genera. IHA was the most sensitive and specific test. Results of phenol/water extraction of the lyophilized *A. fumigatus* extracts, of gel filtration, and of preparative ultracentrifugation indicated that the sensitizing antigen in IHA was a precipitinogen of protein nature with a m.w. of approximately 150,000. The haemolysins in the extracts were probably phospholipases.

Key words: *Aspergillus fumigatus;* antibodies; antigens; haemagglutinogens; haemolysin.

O. Tonder, Broegelmann Research Laboratory for Microbiology, MFH-bygget, 5016 Haukeland sykehus, Bergen, Norway.

Received 19.vii.75 Accepted 19.vii.75

We have recently described indirect haemagglutination as a convenient method for routine demonstration and quantitation of antibodies to *A. fumigatus* (6). Results obtained with rabbit immune serum and human sera indicated that the reaction was specific at higher titres (≧256), but cross-reactions occurred at lower titres.

The purpose of the present work was to compare the results obtained by various serological tests used for demonstration of antibodies to *A. fumigatus* We were particularly interested in studying whether the antigen which sensitizes erythrocytes for agglutination also acts as a precipitinogen, since double diffusion in agar or immunoelectrophoresis are the most widely employed test systems for demonstration of antibodies to *A. fumigatus*. In addition we have performed experiments aimed to characterize the erythrocyte sensitizing antigen in extracts of *A. fumigatus*.

MATERIALS AND METHODS

Aspergillus Strains

The 5 strains of *A. fumigatus* were the same as those used earlier (6): strain Af from this Department, strains 16030, 48338, 131705, and 92877 from Commonwealth Mycological Institute, Kew, Surrey, England. A strain of Aspergillus isolated

in this Department and which could not be species determined was included (Aspergillus 3). *A. pseudoglaucus* and *A. niger* were kindly provided by the Institute of General Microbiology, University of Bergen, and the National Institute of Public Health, Oslo, respectively.

Other Fungi and Bacteria

P. notatum, *C. albicans*, *T. rubrum*, *T. schoenleinii*, *S. aureus*, Micrococcus strains and *E. coli* were selected from routine material.

Erythrocytes

Sheep erythrocytes were obtained from whole blood collected in Alsever's solution. The erythrocytes were handled as earlier described (6).

Sera

Ten sera were selected from the groups of human sera studied earlier (6): Six sera from patients with clinically definite aspergillosis (Br, La, Mt, My, Ne, Pe) were extensively studied in comparison with 4 sera from patients without aspergillosis (Da, Ei, Fr, Or). Sera from blood donors were randomly selected.

A rabbit antiserum to *A. fumigatus* Af was the same as that used earlier (6), and aliquots of the weekly bleedings taken during the immunization were also used.

All sera were heated at 56° C for 30 min before testing to inactivate complement.

Cultures

All fungi, except *C. albicans*, were grown on yeast extract medium and handled as described earlier (6). *C. albicans* was grown on Sabouraud agar plates and the bacteria on nutrient agar plates. The harvested material was washed, homogenized, disrupted in a bacterial press, and lyophilized.

Preparation of Extracts

The lyophilized material was extracted with phosphate buffered saline, pH 7.2 (PBS), as described earlier (6), and concentrations will be given as mg dry weight/ml

Indirect Haemagglutination (IHA) and Inhibition of IHA

Both tests were performed as earlier described (6). Briefly, tanned erythrocytes were sensitized by incubation at 37° C for 1 h in proper concentrations of the extracts: and to 0.025 ml twofold dilutions of serum was added 0.025 ml of sensitized erythrocytes. The haemagglutination was graded as 3+, 2+, 1+ and –, according to the appearance of the pattern of sedimented erythrocytes. The titre is given as the reciprocal of the highest serum dilution which gave agglutination. In inhibition tests, 0.025 ml of extracts of various concentrations was incubated with 0.025 ml of serum before addition of sensitized erythrocytes.

Tests for Haemolysin and Anti-haemolysin

Haemolytic activity of extracts was tested as earlier (6). One haemolytic unit was defined as the smallest amount of serum that gave 50 per cent haemolysis of the 2 per cent sheep erythrocyte suspension. To test anti-haemolysin activity of sera, 0.1 ml of an appropriate dilution of extract containing 3 haemolytic units was added to 0.1 ml dilutions of serum in PBS. After incubation at room temperature for 60 min, 0.1 ml of a 2 per cent suspension of sheep erythrocytes was added. The mixture was incubated at 37° C for 90 min and the degree of lysis recorded. Titre of anti-haemolysin is given as the reciprocal of the highest serum dilution which gave 50 per cent inhibition of lysis.

Precipitation Reactions

The ring precipitation test was performed using 2.5 cm tubes with internal diameter 2 mm (2).

For double diffusion in agar, 1.2 per cent Agar Noble (Difco) in 0.85 per cent saline was employed. The wells had a diameter of 4 mm and were placed 4 mm apart All extracts were made from lyophilized material in concentration 60 mg dry weight/ml. Lines of precipitation were recorded after 48 h at 4° C (5).

Complement Fixation Test (CFT)

Microtitration equipment from CECO (Cooke Engn. Co., Medical Research Division, 800 Slates Lane, Alexandria, Va., USA) was employed. To twofold dilutions of serum in 0.025 ml veronal-buffer (2) was added 0.025 ml of *A. fumigatus* Af extract and 0.05 ml guinea pig serum diluted 1 in 10. After incubation at 4° C for 18 h, 0.05 ml of a 1.5 per cent suspension of sheep erythrocytes sensitized by 4 haemolytic units (50 per cent haemolysis) of rabbit antiserum was added. The degree of lysis was recorded following incubation for 30 min at 37°C. The titre of the serum was given as the reciprocal of the highest original serum dilution which gave 50 per cent inhibition of haemolysis (1).

Absorptions

Sera or extracts at proper dilutions were mixed with equal volumes of packed unsensitized or sensitized tanned erythrocytes. The mixtures were left at 37° C for 1 h and then centrifuged at 1,000 × *g* for 10 min. The supernatants (absorbed serum, absorbed extract) were used in the various tests as will be described.

Extractions with Phenol/Water

Aliquots of 2 g of lyophilized material of *A. fumigatus* Af were extracted following in detail the method described by *Westphal et al.* (7). All final precipitates were lyophilized.

Gel Filtration

To a Sephadex G-200 column, 2.4 × 60 cm, was added 2.5 ml of extract (60 mg/ml), and elution was performed with Tris-buffer (0.05 M Tris-HCl, 0.1–1 M NaCl and 0.02 per cent Na-acid, pH 8). Eluent was collected in 5 ml fractions, and optical density recorded at 254 nm. All fractions constituting one peak were pooled and dialysed against distilled water for 18 h and lyophilized.

Preparative Ultracentrifugation

Extracts (60 mg/ml) were fractionated on a 10 to 40 per cent sucrose gradient by ultracentrifugation at 114,000 × *g* for 18 h. Sixteen fractions, numbered from bottom to top, were collected through a pinhole in the bottom of the tube. A mononucleosis serum containing high titres of IgM antibodies to sheep erythrocytes was run as a control for "serological" localization of 19S and 7S Ig. The fractions were dialysed against PBS before testing.

Phospholipase Activity

The egg yolk test was performed as described by *Lehmann* (3).

RESULTS

Anti-haemolysins

Since many of the extracts of *A. fumigatus* haemolysed various species erythrocytes (6) it was of interest to investigate whether immune serum and patient sera would inhibit this haemolysis. The immune serum (titre in haemagglutination 4096) inhibited the haemolytic activity of extract up to a dilution of 1 in 32, while the patient sera (titres in haemagglutination varying from 16 to 4096) either showed no inhibiting activity or inhibited only at dilution 1 in 2. The results indicated that antibodies to the haemolysin of *A. fumigatus* are not produced in human aspergillosis.

CFT Antibodies

The immune serum gave a titre of 1024 in CFT, but none of the 10 sera from the patients had titres above 16, and 6 sera gave no reaction even undiluted. Further experiments with CFT were therefore not performed.

Precipitins and Haemagglutinins

All bleedings from the immunized rabbits were tested in agar diffusion against extract of *A. fumigatus* Af (60 mg/ml). Pre-immune serum and serum taken 1 week after immunization was started gave no lines. Serum from the other bleedings gave distinct lines, and the number of lines increased after each injection to 6 or 7 following the booster injection.

Extracts of all *A. fumigatus* strains were tested against the booster immune serum. All gave apparently similar number of lines, although the intensity of each line might vary. In this respect there is conformity with IHA, using the various extracts for sensitization of the erythrocytes: Immune serum gave similar titres against all samples of sensitized cells (6).

With a view to further comparison, all bleedings were also tested in IHA. After 1 week of immunization, measurable amounts of antibodies occurred and the maximum level was reached after 3 weeks (Fig. 1). This is in keeping with the general observation that IHA is much more sensitive than precipitation.

Human sera were tested in agar diffusion against extracts of *A. fumigatus*. Fourteen blood donor sera gave no lines, and no reaction in ring test precipitation. Five of the 6 sera from patients with aspergillosis gave 1 to 3 lines, and the 4 sera from patients without aspergillosis gave 1 line. Many different combinations of sera and extract were used, and the results showed that some lines gave reaction of identity between patient sera and immune serum, while other lines were formed with only immune serum or patient serum. Examples of precipitation patterns are shown in Fig. 2. Four of the human sera gave a precipitation line against Aspergillus 3 extract and this line showed identity with the line formed against *A. fumigatus* Af extract. When immune serum was tested against extracts of

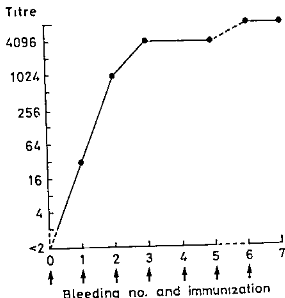


Fig. 1. Titres of serum samples collected during the immunization of a rabbit with extract of *A. fumigatus*. The numbers on the abscissa indicate bleedings at weekly intervals, except 5–6 which was a 3-week period, and arrows indicate injection of extract.

various fungi, patterns like those drawn in Fig. 3 were recorded.

The results indicate that some lines formed with *A. fumigatus* extracts are apparently specific, while other precipitins are cross-

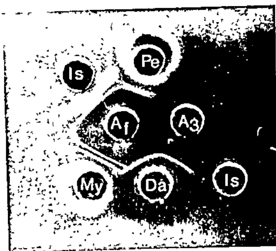


Fig. 2. Double diffusion in agar. Sera from 3 patients (Da, My, Pe) and rabbit immune serum (Is) tested against extract of *A. fumigatus* (Af). An extract of Aspergillus 3 (A3) is included.

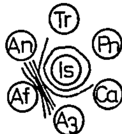


Fig. 3. Double diffusion in agar. Rabbit immune serum to *A. fumigatus* Af (Is) tested against extracts of *A. fumigatus* (Af), *A. niger* (An), Aspergillus 3 (A_3), Trichophyton (Tr), *Penicillium notatum* (Pn), and *Candida albicans* (Ca). Drawing.

reacting with other species and even with other genera of fungi.

Since only extracts of *P. notatum* and Aspergillus 3, in addition to extracts of the 4 *A. fumigatus* strains, and none of the bacterial extracts sensitized erythrocytes for agglutination by immune serum, we designed experiments to investigate whether some of the precipitins acted as haemagglutinins.

Extracts of *A. fumigatus* Af were absorbed with tanned erythrocytes, and absorbed and unabsorbed samples were tested against immune serum. Two lines disappeared after this absorption, the one close to the antigen well and one of the middle lines (cfr. Fig. 3).

That 2 precipitinogens in the extract were absorbed by the tanned erythrocytes corresponded well to the results of absorption of immune serum with sensitized erythrocytes. The same lines disappeared which were not influenced by absorption of immune serum with tanned erythrocytes only.

Serum absorbed with sensitized erythrocytes did not agglutinate erythrocytes sensitized by extract of *A. fumigatus*, but still it agglutinated erythrocytes sensitized by extracts of Aspergillus 3 and *P. notatum*, to titres 128 and 64, respectively. The results indicate that at least 2 precipitating antigen/antibody systems may be involved in haemagglutination.

Nature of Sensitizing Antigens

Extracts were heated at 100° C for 30 min and tested in agar diffusion and IHA together

with unheated extracts. Heated extracts did not sensitize erythrocytes for agglutination by either immune serum or human sera. Again some precipitin lines disappeared, while 2 lines remained with immune serum and one with some of the patient sera. Accordingly, the extract contained both heat labile and heat resistant precipitinogens and at least one of the labile precipitinogens sensitized tanned erythrocytes.

Extracts were treated with phenol/water and the phases tested in agar diffusion and IHA. The water phase gave no precipitation with immune serum and did not sensitize erythrocytes for agglutination. On the other hand, the phenol phase gave one distinct line with immune serum, sensitized erythrocytes optimally and inhibited the agglutination of erythrocytes sensitized by whole extracts. Heated phenol phase preparation was inactive in all 3 tests. Since the phenol phase also gave positive Folin reaction, these results indicate that the sensitizing antigen is of protein nature.

Extracts separated on Sephadex G-200 columns gave 3 elution peaks. Elution volume for the first peak corresponded to that for IgG. The material in this peak sensitized erythrocytes to maximum titre with immune serum, while the second and third peak had no sensitizing activity.

Four of the fractions obtained by preparative ultracentrifugation gave precipitation in agar, but only one of these, fraction 8, inhibited the IHA (Table 1). Accordingly, this activity was well separated from the haemolytic activity appearing in fractions 12 and 13. Heated fraction 8 had no serological activity.

All patient sera which agglutinated erythrocytes sensitized by extracts were tested against the fractions which gave reactions with immune serum. As expected, only those sera which gave highest titres in IHA gave precipitation with fraction 8, while the other fractions reacted with some sera but not with others. On the other hand, sera which precipitated fraction 8 might or might not precipitate some of the other fractions. When fraction 8 was absorbed with tanned erythrocytes, it no longer precipitated with immune serum. These results indicate that the precipitinogen and haemagglutinogen in fraction 8 are identical and that this component is different from the haemolysin, as already suggested above.

TABLE 1. *Fractions Obtained by Preparative Ultracentrifugation of Extract of* A. fumigatus. *Results of Tests for Haemolytic, Precipitating and IHA Inhibiting Activities*

Fraction No.	Haemolysis of SE	Precipitation with antiserum	Inhibition of IHA
1–7	—	—	—
8	—	+	+
9–10	—	—	—
11	—	+	—
12–13	+	+	—
14–16	—	—	—

Localization of markers: IgG: Fractions 7 and 8. IgM: 3 and 4. +: Activity present. —: Activity absent. SE: Sheep erythrocytes.

Extracts and the various fractions of the extracts were tested for phospholipase activity using the egg yolk reaction. Whole extract and ultracentrifugation fractions 12 and 13 had phospholipase activity. These are the same fractions which showed haemolytic activity. Since the haemolysin showed no relation to either precipitinogens or haemagglutinogens, further investigations were not performed.

DISCUSSION

In a recent report (6) we confirmed that extracts of *A. fumigatus* strains haemolysed various species erythrocytes. In the present experiments we tested whether inhibition of the haemolysis could be utilized as a test for antibody activity. Although the rabbit antiserum inhibited the lytic activity, the same did not apply to any of the human sera which were positive in other tests. Accordingly, anti-haemolysin activity cannot be used as an indicator of antibody activity.

Since complement fixation test using the same extracts also gave low titres with human

sera (≷16) we designated our experiments to compare double diffusion in agar and IHA.

Investigations aimed to establish when precipitins and haemagglutinins developed during immunization, showed, as expected, that haemagglutinins could be demonstrated in rapidly increasing titres already 1 week after the first injection of antigen. Maximum titres were reached after 3 weeks. At this time, 2 lines were distinct in agar diffusion, but the number of lines increased to 6 or 7 after the booster dose. This observation clearly shows that the IHA has a high sensitivity at any stage in the development of immunity to *A. fumigatus,* at least with rabbit sera.

In addition, the high sensitivity of IHA appeared to include a high specificity. Althoug immune serum agglutinated erythrocytes sensitized with extracts of *P. notatum* and Aspergillus 3, the titres were low as compared with the titres obtained by erythrocytes sensitized with extracts of *A. fumigatus* strains. Furthermore, among the 9 human sera which gave precipitation, only the 5 which were positive in IHA were from patients with definite aspergillosis, while the other 4 patients in whom IHA was negative did not suffer from the disease. It should also be mentioned that another patient with definite aspergillosis whose serum did not precipitate, agglutinated sensitized erythrocytes to a titre of 256 (6). Accordingly, IHA should be a useful and reliable method in investigations of antibodies to *A. fumigatus.*

That the antigens which are active in IHA have a narrow specificity was also evident from the results of fractionation experiments. Phenol/water extraction yielded precipitinogens both in the phenol and the water phases when tested against immune serum. The phenol phase gave precipitation with sera from patients with aspergillosis, and sensitized erythrocytes for agglutination to high titres by the same sera. The water phase was precipitated by sera from patients with and without aspergillosis, but it did not sensitize erythrocytes for agglutination. In addition, the precipitins in the water phase showed cross-reactions with other aspergilli (Aspergillus 3, *A. niger*) and even with other fungi (Penicillium, Candida, Trichophyton).

Results of separation on Sephadex G-200 column and gradient ultracentrifugation indicate that the sensitizing antigen has a molecular weight of approximately 150,000. The heat lability, the positive Folin test, and the need for tanned erythrocytes in order to achieve sensitization are all in favour of the protein nature of this antigen.

The results have further shown that the haemolytic activity of extracts is connected with components other than the serologically active precipitinogens and haemagglutinogens. The fractions of extracts which showed haemolytic activity had also phospholipase activity as measured by the egg yolk test. These results indicate that the *A. fumigatus* haemolysin may be a phospholipase, thus being similar to many bacterial haemolysins (3, 4). Since apparently no others but the *A. fumigatus* antigens in the extracts which have the highest specificity sensitize erythrocytes, whole extracts can be used for sensitization in routine testing of human sera.

REFERENCES

1. *Biguet, J., Fruit, J., Vernes, A. & Capron, A.:* La réaction de fixation du complément et l'immunoélectrophorèse appliquées au diagnostic immunologique de l'aspergillose pulmonaire. Rev. Immunol (Paris) *34:* 193–203, 1970.
2. *Kabat, E. A. & Mayer, M. M.:* Experimental Immunochemistry, pp. 72, 526, and 557. Charles C. Thomas, Springfield, Ill., 1964.
3. *Lehmann, V.:* Phospholipase activity of *Acinetobacter calcoaceticus.* Acta path. microbiol scand. Sect. B, *79:* 372–376, 1971.
4. *MacFarlane, M. G. & Knight, B. C. J. G.:* The biochemistry of bacterial toxins. I. The lecithinase activity of *Cl. welchii* toxins. Biochem. J. *35:* 884–902, 1941.
5. *Ouchterlony, O.:* Antigen-antibody reactions in gels. Acta path. microbiol. scand. *26:* 507–515, 1949.
6. *Tönder, O. & Rödsæther, M.:* Indirect haemagglutination for demonstration of antibodies to *Aspergillus fumigatus.* Acta path. microbiol. scand. Sect. B, *82:* 871–878, 1974.
7. *Westphal, O., Lüderitz, O. & Bister, F.:* Über die Extraktion von Bakterien mit Phenol/Wasser. Z. Naturforsch. *7b:* 148–155, 1952.

Acta path. microbiol. scand. Sect. C, 83: 429-438, 1975

MITOGENIC RESPONSES OF MOUSE THYMUS AND SPLEEN CELLS EFFECT OF IMMUNOADSORBENT PROCEDURES

M. Hoier-Madsen and Bent Rubin

Immunobiology Laboratory, Statens Seruminstitut, Copenhagen, Denmark

Høier-Madsen, M. & Rubin, B Mitogenic responses of mouse thymus and spleen cells effect of immunoadsorbent procedures. Acta path. microbiol. scand. Sect. C, 83: 429-438, 1975.

The *in vitro* proliferative responses of mouse thymus- and spleen cells against the mitogens phytohaemagglutinin (PHA), concanavalin A (Con A) or lipopolysaccharide (LPS) were determined after either 1) anti-θ antiserum and complement, or 2) immunoadsorbent column fractionation. The responses before and after 1) and 2) were compared with the reduction or enhancement in the number of θ positive cells, EA:RFC or EAC':RFC:s. It was found that the PHA response both of thymus- and spleen cells was equal to or higher after Ig:anti-Ig column fractionation, whereas it was very sensitive to treatment with anti-θ antiserum and complement. The LPS response both of thymus- and spleen cells was very significantly reduced after Ig:anti-Ig column fractionation, but on the other hand, the LPS response of spleen cells was somewhat reduced after anti-θ antiserum and complement treatment. The Con A response of thymus- and spleen cells was very sensitive to anti-θ antiserum and complement treatment, but in addition it was very significantly reduced after passage through Ig:anti-Ig columns, even though the number of θ positive cells increased at least twice and the PHA response was increased. Possible reasons for the latter findings including 1) PHA and Con A stimulate different subpopulations of T cells or 2) a different requirement of adherent cells for the two T cell mitogen *in vitro* responses will be discussed.

Key words: Mitogenic responses; mouse thymus and spleen cells; immunoadsorbent procedures.

M. Høier-Madsen, Immunobiology Laboratory, Blood Grouping Department, Statens Seruminstitut, Amager Boulevard 80, DK-2300 Copenhagen S, Denmark.

Received 21.v.75 Accepted 22.viii.75

Mouse lymphoid cell populations from different anatomical origins contain different proportions of T (thymus-processed) and B (non-thymus-processed) lymphocytes (16). These T and B lymphocytes possess some kind of organ "affinity", i.e. when a given lymphoid cell population is injected into an irradiated individual they migrate preferentially to their organ of origin (10, 12). The T and B lymphocytes in mice can be distinguished either by morphological criteria (presence or absence of surface markers such as the θ antigen (T), the C3 receptor (B), membrane immunoglobulin (Ig) detectable by immunofluorescence (B)) or functional criteria (helper effect, graft-versus-host activity or cytotoxic killer effect (T); antibody forming cell precursors (B)). In addition to the immunologically specific lymphocyte

functions, T and B lymphocytes are stimulated selectively *in vitro* by different mitogens. Thus, mitogens such as phytohaemagglutinin (PHA) and concanavalin A (Con A) in their soluble forms have been shown preferentially to stimulate T lymphocytes, whereas mitogens like pokeweed mitogen (PWM), dextransulphate (DxS) and lipopolysaccharide (LPS) preferentially stimulate B lymphocytes (6, 9, 13).

In studies of the immunological status of immunodeficiency diseases in man, the use of mitogen responses has been of great importance for the delineation of the lymphocyte class of the immunodeficiency (1, 11, 13, 17). Selective mitogen responses (6) have also been used in studies of the maturation of B and T lymphocytes from their precursors in the bone marrow of foetal liver. We have studied the development of B and T lymphocytes from bone marrow cells either in semi-allogeneic versus syngeneic combinations (25) or after filtration of the bone marrow cells through different types of affinity columns (22). In the latter studies we observed that passage of bone marrow cells through Ig: anti-Ig coated columns selectively removed precursor cells of GVH and helper cells, both of which are T lymphocyte functions. It was further shown that the active principle on such columns was the presence of either an antigen-antibody complex and an anti-Ig activity, or both. The purpose of the present study was to establish an *in vitro* system in which we could measure specific T and B lymphocyte functions by means of mitogens. Such a system would be a very effective means of studying the development of T and B lymphocytes from bone marrow cells which were subjected to affinity column fractionation procedures.

MATERIALS AND METHODS

Animals: Inbred mice of the following genotype were used throughout the experiments: C57Bl/6 (H-2^b), Balb/c (H-2^d), C3H and AKR/J (H-2^k), CBA × Balb/c (H-2^k/H-2^d) and ASW × CBA (H-2^s/H-2^k). They were bred in our own colony and were 6–8 weeks old when used.

Mitogens: Purified phytohaemagglutinin (PHA) was obtained from Wellcome Research Laboratories (HA17 lot No. 8805), concanavalin A (Con A) was obtained from Pharmacia, Uppsala, Sweden (lot No. 3059) and *E. coli* O55:B5 lipopolysaccharide (LPS) from Difco Laboratories (lot No 503309). Stock solutions of each mitogen in sterile saline contained 2 mg/ml.

Cultures: Eagle's minimum essential medium F13 (Gibco) supplemented with 100 I.U. of penicillin/ml, 50 μg of streptomycin/ml, 2 mM glutamine, and non-essential amino acids (100 ×, Flow Laboratories diluted 1:100) was used as tissue culture medium. In addition, the medium contained 10 per cent foetal calf serum (FCS) (Flow Laboratories lots No. 42177, 470055 and 415075).

Cell suspensions were made aseptically from thymus and spleen tissue. The cells were washed twice in 10 per cent FCS/F13 and the number of trypan blue excluding cells was determined. The cell concentration was adjusted to 10 times the desired concentration. 0.1 ml of this stock solution was added to Falcon plastic tubes (No. 2001) pre-filled with 1.8 ml 10 per cent FCS/F13 and 0.1 ml of mitogen. Mitogen responses were determined in triplicates. All cutures were incubated at 37° C in a humified atmosphere containing 5 per cent CO_2. Cell and mitogen concentrations as well as incubation times will be described under Results.

Cytotoxicity: Anti-θ antiserum was prepared by immunizing female AKR/J mice with female C3H/J thymocytes. One week after the last of six weekly injections the AKR mice were bled, the serum was isolated, heat-inactivated (30 min at 56° C), sterile filtered, and stored at −30° C. 10^8 spleen cells were resuspended in 2 ml of anti-θ antiserum or 2 ml of 10 per cent FCS/F13. They were incubated for 1 hour at 4° C and then centrifuged. The cell pellet was resuspended in 5 ml normal guinea pig serum diluted 1:5 in 10 per cent FCS/F13. These suspensions were incubated for 45 minutes at 37° C, centrifuged and the cells were resuspended in 5 ml of 10 per cent FCS/F13. The number of trypan blue excluding cells was determined. Normal spleen cells treated in this way contained 32.5 ± 5.8 θ positive cells (one standard deviation). The specificity of the anti-θ antiserum was controlled by the following criteria: 1) it killed 50 per cent of CBA thymocytes at a dilution of 1:256, 2) it did not kill AKR thymocytes, 2) its activity could be adsorbed completely by CBA brain tissue but not by AKR brain tissue, and 4) it killed less than 5 per cent of bone marrow cells at any dilution, whether or not the bone marrow cells had been passed through Ig:anti-Ig columns (25).

Rosette assays: EA- and EAC'-RCS s were determined as described previously (24). E = sheep red cells (SRBC); A = anti-SRBC antibody; C =

mouse complement. A for EA complexes was 7S mouse anti-SRBC; for EAC complexes = 19S rabbit anti-SRBC.

Affinity columns: Glass bead columns were prepared as described previously (19, 26). The glass beads (200 μ in diameter) used in the present study were obtained from Dr. *H. Wigzell*, Uppsala, Sweden (3M, USA). The following types of columns were prepared: 1) Mouse immunoglobulin (MIg) coated columns (designated Ig columns), 2) human serum albumin (HSA) coated columns (designated HSA columns), 3) HSA:anti-HSA complex columns (designated HSA:anti-HSA columns), and 4) Ig:anti-Ig complex columns (designated Ig:anti-Ig columns). Sterility was achieved by boiling the glass beads, treating the glass columns with 50 per cent ethanol/water overnight, and sterile filtration of the protein solutions used (19).

In addition we used degalan plastic bead columns coated with either normal rabbit globulin (designated NRG columns) or affinity column purified rabbit anti-MIg antibodies (designated anti-Ig columns). These columns were kept sterile by storing the degalan beads in sterile saline containing 0.2 per cent sodium azide and by thoroughly washing the beads with 10 per cent FCS/F13 before cell passage (at least 100 ml were passed through the beads). Flow rate was in all cases 2-5 ml per minute and the cells were passed through the columns at room temperature.

Statistics: The cultures were made in triplicate as described. Geometric means and SD (standard deviation) or SE (standard error) were calculated (27). The results will be given as 1) log cpm ± SD, or 2) Δ cpm = anti-log to the geometric mean of mitogen stimulated cultures minus anti-log to the geometric mean of control cultures without mitogen. All cpm values are given as cpm per culture. The students t test was used to determine the degree of significance with an arbitrary level of significance of $P \leq 0.01$ (if necessary at all).

RESULTS

Comments on the Experimental Design and on the Culture System

The present study was performed in order to determine whether T lymphocytes were selectively stimulated by the mitogens PHA and Con A, and B lymphocytes were selectively stimulated by LPS in our tissue culture system (13). Two methods were used to enrich thymus- and spleen cells for T and B lymphocytes, respectively: 1) Passage through Ig:anti-Ig columns and 2) treatment with anti-Θ antiserum and complement. One criterium for regarding PHA and Con A as T lymphocyte stimulants and LPS as a B lymphocyte stimulant is that the PHA or Con A responses of Ig:anti-Ig column passed cells should be equal to or higher than that of non-passed cells and that the LPS response of Ig:anti-Ig column passed cells should be reduced by more than 90 per cent when compared with non-passed cells. A second criterium is that the PHA or Con A responses of anti-Θ antiserum and complement treated cells should be reduced by more than 90 per cent when compared with the response of cells treated with 10 per cent FCS/F13 and complement, whereas the LPS response of anti-Θ antiserum and complement treated cells should be equal to or higher than the response of cells treated with 10 per cent FCS/F13 and complement. Objections to these criteria will be discussed in the Discussion section on the basis of the results obtained.

The culture system used was adopted from our previous *in vitro* studies of guinea pig cells (19), see also Materials and Methods. Experiments with thymus- and spleen cells stimulated with PHA, Con A and LPS

TABLE 1. *Mitogen Responses of Normal Balb/c Thymus Cells*

Thymus cells/ml	Mitogen/ml	^{3}H-thymidine incorporation $\log_{10}$cpm ± SD*	Δcpm*
5×10^6	–	2.541 ± 0.150	–
"	100 μg PHA	2.895 ± 0.025	437
"	10 " "	4 200 ± 0 049	15,552
"	1 " "	3.390 ± 0.049	2,012
"	0.1 " "	2.423 ± 0.101	0
5×10^6	100 μg Con A	2.385 ± 0.244	0
"	10 " "	4.285 ± 0.085	18,952
"	1 " "	4.417 ± 0.101	25,752
"	0.1 " "	2.448 ± 0.125	0
5×10^6	100 μg LPS	3.085 ± 0.063	872
"	10 " "	3.232 ± 0.015	1,362
"	1 " "	2.885 ± 0.168	420
"	0.1 " "	2.599 ± 0.188	49

* SD = one standard deviation; Δ cpm = cpm in mitogen stimulated cultures minus cpm in non-stimulated cultures, calculated as described in Materials and Methods Section.

showed that, in our system, the optimal culture time was 3 days, the last 18 hours being in the presence of tritiated thymidine as found by others (13). The optimal mitogen concentrations of thymus cells was found to be 10 μg PHA/ml, 1 μg Con A/ml and 10 μg LPS/ml (Table 1) and that of spleen cells 1 μg PHA or Con A/ml and 10 μg LPS/ml. These figures are also in agreement with those obtained by others (13). Since one purpose of the present study was the design of a culture system in which we could quantitate the number of mitogen reactive cells, it was important to find a lymphocyte concentration range where the mitogen responses were directly proportional to the number of lymphocytes cultured. Therefore, we investigated the lymphocyte dose responses of thymus- and spleen cells. As can be seen in the different experiments presented in this study, the dose responses of thymus cells varied. In summary, the PHA response seems to increase with the cell concentration up to and probably beyond 10×10^6 cells/ml, whereas the optimal response to Con A was obtained with about 2.5×10^6 cells/ml. Usually, thymus cells gave a weak but significant response to LPS, and the optimal response was obtained with about 5×10^6 cells/ml (Table 5). In contrast, spleen cells gave optimal responses to all three mitogens at a cell concentration of about 1×10^6 cells/ml (Table 2) and relatively linear dose response curves were obtained in the range of 1×10^5 cells/ml to 1×10^6 cells/ml (Fig. 1) when the results were plotted on a log-log basis.

TABLE 2. *Mitogen Responses of Normal Balb/c Spleen Cells*

Spleen cells/ml	Mitogen/ml	^{3}H-thymidine incorporation	
		$\log_{10}$cpm ± SD*	Δcpm*
3×10^6	–	3.854 ± 0.067	–
1×10^6	–	3.476 ± 0.091	–
3×10^5	–	2.923 ± 0.062	–
3×10^6	1 μg PHA	5.023 ± 0.086	98,255
1×10^6	„	4.937 ± 0.093	83,501
3×10^5	„	4.616 ± 0.067	40,397
3×10^6	1 μg Con A	4.853 ± 0.038	64,145
1×10^6	„	4.754 ± 0.021	53,751
3×10^5	„	4.247 ± 0.031	16,757
3×10^6	10 μg LPS	4.357 ± 0.012	15,605
1×10^6	„	4.237 ± 0.031	14,261
3×10^5	„	3.701 ± 0.040	4,121

* See Table 1.

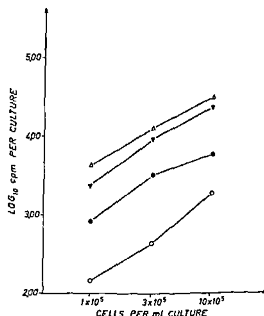


Fig. 1. Mitogenic responses of mouse spleen cells. ○—○ = no mitogen; ●—● = 10 μg LPS/ml; ▼—▼ = 1 μg Con A/ml; △—△ = 1 μg PHA/ml; Culture conditions, see text.

Effect of Anti-O Antiserum and Complement on the Mitogenic Responses of Mouse Spleen Cells

A total of five individual experiments showed that the mitogenic response of spleen cells to PHA and Con A was highly sensitive to the action of anti-O antiserum and complement (Table 3), but not to the action of complement or anti-O antiserum. However, the mitogenic response of spleen cells to LPS was also somewhat sensitive to anti-O antiserum and complement treatment, but not to either complement or anti-O antiserum alone. The degree of reduction in the LPS response varied with different concentrations of either the mitogen (Table 3) or the cells. If the LPS response of splenic lymphocytes was completely independent of the presence of

TABLE 3. *Effect of Anti-Θ Antiserum and Complement Treatment of Normal Balb/c Spleen Cells on Their in* Vitro *Mitogenic Responses*

Spleen cells per ml	Mitogen per ml	^{3}H-thymidine incorporation Δ cpm* C	anti-Θ + C	% of control§	% of Expect.†
1×10⁶	100 µg LPS	3,916	1,664	42.5	
„	10 „ „	5,556	3,904	70 4	
„	1 „ „	7,662	3,107	40.8	137
„	0.10 „ „	4,345	2,024	46.6	
„	0.01 „ „	2,010	924	46 0	
1×10⁶	10 µg PHA	39	1,904	–	
„	1 „ „	23,531	50	0.21	0.0
„	0.1 „ „	22.851	417	1.82	

* See Table 1.

§ Δ cpm of anti-Θ antiserum and complement treated cells as percentage of Δ cpm of complement treated cells.

† % Θ positive cells = 37.1; EAC'-RFC:s before and after anti-Θ antiserum and complement = 19 8% and 28.3%, respectively

T lymphocytes, the LPS response after anti-Θ antiserum and complement treatment should have been about 135 per cent of that found after complement treatment, due to the expected enrichment in B lymphocytes after anti-Θ antiserum and complement treatment (Table 3).

Effect of Column Fractionation on the Mitogenic Responses of Mouse Spleen Cells

Spleen cells either non-passed or passed through Ig-, HSA:anti-HSA- or Ig:anti-Ig columns were tested for their mitogenic responses to PHA, Con A or LPS. As expected, the LPS response of spleen cells and their ability to make EA- and EAC-RFC was highly reduced following passage through Ig:anti-Ig columns, and the PHA response of spleen cells was somewhat increased after passage through any of the columns used (14). In contrast, the Con A response of spleen cells was usually somewhat reduced after passage through control columns and very significantly reduced after passage through Ig:anti-Ig columns. Exp. 1 in Table 4 is an example of the results obtained in three experiments, and Exp 2 in Table 4 shows the result of one experiment. The last three experiments (of the seven experiments performed so far) showed reduction in the Con A response after Ig:anti-Ig passage in between the examples given in Table 4. It should be noted that the optimal concentrations of PHA and Con A are the same before and after column passage.

Effect of Column Fractionation on the Mitogenic Responses of Mouse Thymus Cells

Although great care was taken to remove subcapsular lymph nodes, our thymus cells always gave a weak response to LPS. This response may be due to the presence of small amounts of B lymphocytes in the thymus cell preparation, as indicated in the results in Tables 5 and 6. The LPS response is selectively removed on Ig:anti-Ig columns. The PHA response of thymus cells is usually the same or higher after passage through Ig:anti-Ig columns, compared with the situation after passage through control columns or if non-passed. However, the Con A response of thymus cells was found to be severely reduced after passage through Ig:anti-Ig columns, compared with the response of non-passed cells (Table 5) or with the response after passage through control columns (Table 6).

TABLE 4. *Effect of Column Passage of Normal Mouse Spleen Cells on Their in Vitro Mitogenic Responses*

Mitogen per ml	^{3}H-thymidine incorporation (Δ cpm)*					
	Exp. no. 1§			Exp. no. 2§		
	Non-P	Ig-P	Ig:anti-Ig:P	Non-P	HSA:anti-HSA:P	Ig:anti-Ig:P
1 μg PHA	81,532	103,402	100,154	41,740	91,225	91,623
1 „ Con A	64,820	38,814	9,089	75,780	96,110	53,139
10 „ LPS	28,256	26,734	1,035	41,216	65,359	1,921

* 10^6 spleen cells/ml (CBA × Balb/c); Δ cpm = see Table 1.

§ Exp. no.	Recovery (%)	EA-RFC (% red.)	EAC-RFC (% red.)
1 (Ig-P)	24.2	39.0	13.8
(Ig:anti-Ig:P)	21.8	94.3	96.3
2 (HSA:anti-HSA:P)	46.2	70.5	56.0
(Ig:anti-Ig:P)	13.8	96.2	95.8

red. = reduction.

TABLE 5. *Effect of Ig:anti-Ig Column Passage of Normal Balb/c Thymocytes on Their* in Vitro *Mitogenic Responses*

Cells per ml	Mitogen per ml	^{3}H-thymidine incorporation (Δcpm)*		% of control
		Non-P	Ig:anti-Ig:P§	
10 × 10^6	10 μg PHA	53,517	25,731	48.0
5 × 10^6	„	19,050	20,430	107
2.5 × 10^6	„	2,215	5,741	259
10 × 10^6	1 μg Con A	12,797	1,408	11.0
5 × 10^6	„	27,085	3,645	13.5
2.5 × 10^6	„	32,368	6,764	20.9
10 × 10^6	10 μg LPS	643	0	0
5 × 10^6	„	871	0	0
2.5 × 10^6	„	99	0	0

* See Table 1.
§ Recovery after passage = 57.8 %, Reduction in EA-RFC = 97.3 %.

TABLE 6. *Effect of Passage Through Different Kinds of Columns on the* in Vitro *Mitogenic Response of Normal Thymocytes*

Thymus cells per ml	Mitogen per ml	^{3}H-thymidine incorporation (Δ cpm)*			
		Non-P	Ig-P§	HSA:anti-HSA:P§	Ig:anti-Ig:P§
5 × 10^6	10 μg PHA	2,805	5,846	2,962	2,032
2 × 10^6	„	704	583	1,821	675
5 × 10^6	1 μg Con A	11,532	9,626	9,588	1,840
2 × 10^6	„	6,091	8,266	5,019	243
5 × 10^6	10 μg LPS	342	218	195	0
2 × 10^6	„	136	622	128	0

* See Table 1.
§ Recovery: Ig-P = 89.1 %, HSA anti-HSA P = 74.6 %; Ig:anti-Ig:P = 78.2 %.

TABLE 7. *Effect of Anti-Ig Column Passage on the Mitogenic Responses of Normal Mouse Thymus Cells*

Cells per ml	Mitogen per ml	^{3}H-thymidine incorporation (Δ cpm)* Non-P	NRG-P§	anti-Ig:P§
5×10^6	10 µg PHA	6,519	7,736	9,281
2×10^6	"	736	658	606
5×10^6	1 µg Con A	6,054	5,045	2,975
2×10^6	"	6,900	4,894	4,176
5×10^6	10 µg LPS	356	344	0
2×10^6	"	156	117	0

* See Table 1. Mouse = ASW × CBA.
§ Recovery: NRG-P = 91.8 %; anti-Ig:P = 74.6 %.
Reduction in EA-RFC:s: NRG-P = 58.6 %; anti-Ig:P = 27.4 %.

TABLE 8. *Effect of Anti-Ig Column Passage on the Mitogenic Responses of Normal Mouse Spleen Cells*

Spleen cells per ml	Mitogen per ml	^{3}H-thymidine incorporation (Δ cpm)* Non-P	NRG-P§	anti-Ig:P§
10×10^5	1 µg PHA	16,047	31,248	77,898
3×10^5	"	9,838	15,249	25,638
10×10^5	1 µg Con A	25,645	37,315	36,347
3×10^5	"	9,399	16,537	17,406
10×10^5	10 µg LPS	40,085	40,836	3,443
3×10^5	"	3,893	5,304	0
θ positive cells (%)		36.5	67.2	90.3
EA-RFC:s (%)		26.0	21.2	2.5
EAC-RFC:s (%)		24.0	18.8	0.92

* See Table 1. Mouse = ASW × CBA.
§ Recovery: NRG-P = 55.5 %; anti-Ig:P = 22 8 %.

Effect of Anti-Ig Columns on the Mitogenic Response of Mouse Spleen and Thymus Cells

The most important finding from the present experiments would seem to be that the Con A response both of thymus- and spleen cells is very significantly reduced after passage through Ig:anti-Ig columns. Therefore, we determined whether columns coated with purified anti-Ig antibodies would also reduce the Con A response of thymus and spleen cells. The Con A response of thymus cells was significantly reduced after passage through anti-Ig columns in most experiments, but the effect was not as striking as that seen after Ig:anti-Ig columns passage (Table 7). This finding was substantiated by the results obtained with spleen cells. The Con A response of spleen cells was not reduced; on the contrary, it was enhanced after anti-Ig columns passage (Table 8). One difference between the experiments in this section and those of the previous sections is that purified anti-Ig antibodies were coated on to degalan plastic beads, whereas the other columns were made of glass beads. This was done because it has been found that plastic beads bind somewhat more protein as compared with glass beads (*Wigzell*, personal communication).

DISCUSSION

The present results have shown that it was possible to establish an *in vitro* system ir-

which we could measure specific T or B lymphocyte functions by means of mitogens. However, we would like to stress from the outset that the only *in vitro* mitogenic response which complies with our criteria for the evaluation of the fractionation results was the PHA response both of thymus- and spleen cells. This response was highly sensitive to the action of anti-O antiserum and complement and cell populations passed through the different kinds of affinity columns used showed an increased PHA response. Thus, we could classify the PHA response as an autonomous T lymphocyte response and use the PHA response as a marker of T cell function (8, 13).

With respect to the Con A or LPS responses, the situation was less clear. It applies to both responses that one necessary criterium was fulfilled, *i.e.* the Con A response was very sensitive to anti-O antiserum and complement treatment, and the LPS response was significantly reduced after Ig:anti-Ig or anti-Ig column passage. However, the LPS response of spleen cells was somewhat reduced after treatment with anti-O antiserum and complement when compared with the response of complement treated spleen cells. It was significantly reduced, taking into consideration the enrichment in B cells seen after anti-Θ antiserum and complement treatment (Table 3). Therefore, either our criteria were wrong or the LPS response is not a pure B cell mitogenic response. One possible explanation would be that the LPS response is dependent on adherent cells, but it has been proved that this is not the case (6, 13), and the results of our control column experiments would not support such a hypothesis. Another explanation might be that LPS stimulates some subpopulation of T lymphocytes and there is some support for such an alternative in that thymus cells always give a weak but significant response to LPS (and contain 1–3 per cent Fc receptor bearing cells (Tables 5–7)). However, if the latter observation is correct, then the T cells responding to LPS, *in vitro,* in addition to the O antigen should carry immunoglobulin on their surface (the LPS response of thymus cells is completely removed by Ig:anti-Ig or anti-Ig columns passage). A third possibility would be that the induction of the LPS response by B cells is dependent on T cells. Since antibody-forming cell precursors (B cells) *in vivo* are also dependent on T cells during certain stages in their development into antibody-forming plasma cells (3, 5, 18), we regard the present results as being most compatible with the third possibility and we would classify the *in vitro* LPS response as an effector B lymphocyte response.

The Con A response both of thymus- and spleen cells, although very sensitive to anti-O antiserum and complement, was significantly reduced after Ig:anti-Ig column passage. Therefore, we studied the active principle(s) on such columns in detail. Ig:anti-Ig columns could absorb lymphocytes due to either 1) adherent properties, 2) the presence of an antigen-antibody complex, or 3) the presence of the anti-Ig activity. As regards 1), the Con A response of both thymus- and spleen cells seems to be somewhat, but not always, dependent on adherent cells (Tables 5–7) (14). The same holds true of the possibility that the active principle is an antigen-antibody complex. Thus, one might be tempted to conclude that the active principle is the anti-Ig activity. In order to verify this conclusion we carried out fractionation on degalan plastic bead columns coated with either the IgG fraction of normal rabbit globulin, or affinity chromatography purified anti-Ig antibodies. In this case, the Con A response of thymus cells was only marginally reduced after passage through anti-Ig columns (Table 7) and the Con A response of spleen cells was enhanced rather than reduced after anti-Ig column passage (Table 8).

Thus, we are left with the fact that there is a very significant difference between the fractionation of thymic or splenic lymphocytes on anti-Ig columns and on Ig:anti-Ig columns, as regards their Con A response. This difference is probably *not* solely due to the presence of an antigen-antibody complex. The different bead materials used have been

touched upon already, but control columns proved that this difference was insignificant. However, certain T cells might have small amounts of immunoglobulin on their surface in addition to Fc receptors of low affinity. Thus, these cells may be preferentially adsorbed on to columns which express both anti-Ig activity and an antigen-antibody complex (4, 7, 15). Cells with similar characteristics have previously been demonstrated: 1) precursor T cells in the bone marrow (22) and 2) suppressor T cells (21, 23). As regards 1), the Con A responding cells among thymocytes may be less mature than PHA responding cells (9, 13), and they are present in higher numbers as compared with PHA responding cells (Table 5). As regards 2), thymus cells passed through Ig:anti-Ig columns showed increased helper capacity in the induction of a primary anti-sheep erythrocyte (SRBC) antibody response when mixed with normal bone marrow cells and injected with SRBC into lethally irradiated mice (by analogy with the results obtained by passing thymocytes through histamine coated columns (24)). Mixed lymph node and spleen cells from HSA or OA (ovalbumin) immunized mice showed increased helper capacity if passed through either Ig:anti-Ig columns or antigen-specific columns, but no additional increase was observed if the helper cells were first passed through Ig:anti-Ig columns and then through antigen-specific columns or *vice versa* (23). These results indicate that suppressor T cells (if they are T cells; but they are at least sensitive to anti-Θ antiserum and complement) express both Fc receptors and antigen-specific receptors (Ig?). Another puzzling phenomenon regarding suppressor cells in thymocyte populations is the observation that thymocytes adsorbed onto fibroblast monolayers frequently showed a non-specifically enhanced GVH response when injected into newborn F_1 hybrid mice (20).

In conclusion, the Con A response of mouse thymus- and spleen cells is elicited by Θ positive cells which appear to have characteristics similar to Θ positive suppressor cells, *i.e.* 1) somewhat adherent, 2) expressing Fc receptors of relatively low affinity, and 3) expressing low or variable amounts of surface immunoglobulin. Alternative explanations would be that the Con A response is dependent on a collaboration between two subsets of T cells as in the *in vivo* GVH response (2, 20), or on certain critical amounts of adherent cells, the presence of which varies even under the present strictly controlled cell preparations or column fractionation procedures. However, whatever the explanation, Con A responding cells will be looked for with considerable interest in a study of the development of T and B lymphocyte functions from bone marrow cells in lethally irradiated mice.

The authors thank Dr. *J. M. Rhodes* for reading the manuscrips and Dr. *J. Andersen* for providing the opportunity to carry out the present work. It is a pleasure to acknowledge the skilful technical assistance of *J. Lieberkind* and *B. Rumler* and the expert secretarial assistance of *A. Thorborg*. We also wish to thank Mr *E. Christensen* for breeding our mice. This work was supported by grants (to B. R.) from *Statens Lægevidenskabelige Forskningsråd* (512-2642 and 512-3506).

REFERENCES

1. *Aiuti, F. & Wigzell, H.*: Function and distribution pattern of human T lymphocytes. I. Production of specific anti-T lymphocyte sera as established by cytotoxicity and elimination of function of lymphocytes. Clin. exp. Immunol. *13*: 171–179, 1973.
2. *Cantor, H. & Asofsky, R.*: Synergy among lymphoid cells mediating the graft-versus-host response. II. Synergy in graft-versus-host reaction produced by Balb/c cells of different anatomic origin. J. exp. Med. *131*: 235–251, 1970.
3. *Cooper, M. D., Lawton, A. R. & Kincade, P. W.*: A developmental approach to the biological basis for antibody diversity. In. Hanna, M. G. (Ed.): Contemporary Topics in Immunobiology, Plenum Press, N.Y. 1972, p. 33.
4. *Crone, M., Koch, C. & Simonsen, M.*: The elusive T cell receptor. Transplant. Rev. *10*: 33–52, 1972.
5. *Davie, J. M. & Paul, W. E.*: Role of T lymphocytes in the humoral immune response. I. Proliferation of B lymphocytes in thymus-deprived mice. J. Immunol. *113*: 1438–1447, 1974.

6. *Gronowicz, E., Coutinho, A.* & *Möller, G.:* Differentiation of B cells. Sequential appearance of responsiveness to polyclonal activators. Scand. J. Immunol. *3:* 413–423, 1974.

7. *Hudson, L., Sprent, J., Miller, J. F. A. P.* & *Playfair, J. H. L.:* B-cell derived immunoglobulin on mouse activated T lymphocytes. Nature (Lond.) *251:* 60–62, 1974.

8. *Jacobsson, H.* & *Blomgren, H.:* Autonomy of the PHA-responsive cells in the mouse thymus. Scand. J. Haematol. *12:* 123–132, 1974.

9. *Jacobsson, H.* & *Blomgren, H.:* Responses of thymic cells to mitogens. A comparison between phytohemagglutinin and concanavalin A. Cell. Immunol. *11:* 427–441, 1974.

10. *Lance, E. M.* & *Taub, R. N.:* Segregation of lymphocyte populations through differential migration. Nature (Lond.) *221:* 841–844, 1969.

11. *Meuwissen, H. J., Bach, F. H., Hong, R.* & *Good, R. A.:* Lymphocyte studies in congenital thymic dysplasia: The one-way stimulation test. J. Pediat. *72:* 177–183, 1968.

12. *Morse, H. C. III* & *Asofsky, R.:* In vivo effects of anti-thymocyte serum on the homing patterns and graft-versus-host reactivity of murine splenic lymphocytes. Cell. Immunol. *11:* 13–29, 1974.

13. *Möller, G. (Ed.):* Lymphocyte activation by mitogens. Transplant. Rev. *11:* 1972.

14. *Mugraby, L., Gery, I.* & *Sulitzeanu, D.:* Subpopulations of mouse spleen lymphocytes. I. Dissociation between the reactivities to different "non-specific" mitogens. Immunology *26:* 787–795, 1974.

15. *Playfair, J. H. L.:* The role of antibody in T cell responses. Clin. exp. Immunol. *17:* 1–8, 1974.

16. *Raff, M. C.:* Surface antigenic markers for distinguishing T and B lymphocytes in mice. Transplant. Rev. *6:* 52–75, 1971.

17. *Rocklin, R. E., Rosen, F. S.* & *David, J. R.:* In vitro lymphocyte response of patients with immunologic deficiency diseases. New Engl. J. Med. *282:* 1340–1351, 1970

18. *Rubin, B., Hiesche, K., Schirrmacher, V.* & *Wigzell, H.:* The immune response against hapten-autologous protein conjugates in the mouse. IV. Thymus dependency of the primary response to hapten-autologous albumin conjugates. J. Immunol. *111:* 492–499, 1973.

19. *Rubin, B.* & *Wigzell, H.:* Lymphocyte proliferation in vitro induced by hapten-autologous protein conjugates. I. A study on the class of lymphocytes responding in vitro and on the nature and specificity of their receptors. J. exp. Med. *139:* 732–753, 1974.

20. *Rubin, B.:* Studies on the adsorbability of graft-versus-host reactive lymphocytes. Clin. exp. Immunol. *20:* 509–522, 1975.

21. *Rubin, B.* & *Hertel-Wulff, B.:* The biological significance of Fc receptor bearing cells among activated T lymphocytes. Scand. J. Immunol. *4:* 451–462, 1975.

22. *Rubin, B., Trier, L., Hertel-Wulff, B.* & *Rummler, B.:* Selective retension of T cell precursors in bone marrow cell populations after filtration through anti-immunoglobulin columns. Cell. Immunol. *16:* 315–329, 1975.

23. *Rubin, B.:* Regulation of helper cell activity by specifically adsorbable T lymphocytes. J. Immunol. 1976 (in press).

24. *Shearer, G. M., Weinstein, Y.* & *Melmon, K. L.:* Enhancement of immune potential of mouse lymphoid cells fractionated on insolubilized conjugated histamin columns. J. Immunol. *113:* 597–607, 1974.

25. *Trier, L.* & *Rubin, B.:* Bone marrow transplantation in inbred strains of mice. I. The failure of development of normal T cell function following allogeneic transplantation. Acta path. microbiol. scand. Sect. B, *82:* 724–732, 1974.

26. *Wigzell, H., Sundquist, K. G.* & *Yoshida, T. O.:* Separation of cells according to surface antigens by the use of antibody-coated columns. Fractionation of cells carrying immunoglobulins and blood group antigens. Scand. J. Immunol. *1:* 75–87, 1972.

27. *Ziegler, J. B., Hansen, P. J, Davies, W. A.* & *Penny, R.:* The PHA dose-response curve: validation of the use of logarithmic graph paper by computer analysis results. J. Immunol. *113:* 2035–2039, 1974.

Acta path. microbiol. scand. Sect. C, *83:* 439–446, 1975

AMYLOID OF HUMAN ISLETS OF LANGERHANS

1. Isolation and Some Characteristics

PER WESTERMARK

Department of Pathology, University of Uppsala, Uppsala, Sweden

Westermark, P. Amyloid of human islets of Langerhans. 1. Isolation and some characteristics. Acta path. microbiol. scand. Sect. C, *83:* 439–446, 1975

Islet amyloid concentrates containing a maximum of 50 per cent of amyloid fibrils as estimated by light microscopy were obtained from pancreases of patients with maturity onset diabetes mellitus. When centrifuged, the amyloid did not form a top layer as do most other amyloids, but was found in the bottom layer. This phenomenon and the difficulty in dissolving islet amyloid seemed to be due to inability to separate the amyloid fibrils from each other. In SDS electrophoresis, one or two weak bands corresponding to a molecular weight of about 3000–4000 daltons were observed, but most of the material did not enter the polyacrylamide gels. Spectrophotometrically, no tryptophane and almost no tyrosine were demonstrated. It is concluded that the major islet amyloid protein differs fundamentally from other characterized amyloid proteins and, probably together with amyloid of medullary carcinoma of the thyroid, forms a new class of amyloid proteins.

Key words: Amyloid; human islets of Langerhans

Per Westermark, Department of Pathology, P.O. Box 553, S-751 22 Uppsala, Sweden.

Received 26.vi.75 Accepted 26.vi.75

During the last five years, an increasing number of papers dealing with the physical and chemical properties of amyloid have been published. This has been due to the introduction of a simple and reliable method by which to obtain almost pure amyloid fibrils (*Cohen & Calkins* 1964, *Pras et al.* 1968 and 1969) and the finding that the fibrils can be dissolved in strong denaturing agents such as urea or guanidine hydrochloride (*Glenner et al.* 1969, *Benditt & Eriksen* 1971, *Harada et al.* 1971, *Glenner et al.* 1972). It has been found that the major proteins of amyloid fibrils of patients with primary or myeloma-associated amyloidosis vary in composition, but usually seem to be part of the variable segments of immunoglobulin light chains (*Glenner et al.* 1970a and b, *Glenner et al.* 1971). The major protein of fibrils of secondary amyloids seems to vary very little and is a non-immunoglobulin protein, unknown until recently (*Benditt & Eriksen* 1971, *Benditt et al.* 1971, *Ein et al.* 1972, *Husby et al.* 1972, *Levin et al.* 1972, *Pras & Reshef* 1972). This protein has now also been found in other amyloids (*Husby et al.* 1973a and b). Recently, a third group of amyloid fibril proteins has been described (*Husby et al.* 1974) which, however, seems to be part of an unusual light chain (*Sletten et al.* 1974).

All these studies have been performed on isolated amyloid fibrils obtained from ...

TABLE 1. *Patients with Diabetes Mellitus and Severe Islet Amyloidosis*

Case No.	Sex	Age at death (years)	Duration of diabetes (years)
Is 42	F	81	<1
Is 46	M	76	<1
Is 62	F	79	10
Is 74	F	72	unknown
Is 84	F	76	4
Is 100	F	74	1
Is 105	M	68	unknown

with very heavy amyloid deposits usually occupying 50 per cent or more of the organ. However, deposition of small amounts of amyloid may be a cardinal finding in some diseases. The best known example is amyloidosis of the islets of Langerhans in maturity onset diabetes (*Opie* 1900, *Bell* 1952, *Warren et al.* 1966). The deposition of amyloid in the affected organ, i.e. the pancreatic islets, is often very heavy, but since the islets are scattered in the pancreas and only constitute 2–3 per cent of the total gland, studies of the composition of islet amyloid are difficult.

In a previous paper, the occurrence of P-component in islet amyloid has been reported (*Westermark et al.* 1975). The present paper reports a method of isolation of islet amyloid fibrils and some properties of these fibrils.

MATERIAL AND METHODS

Tissues

Pancreases from patients with maturity onset diabetes were obtained within 24 hours after death. As soon as the abdomen was opened, the tail and the body of the pancreas were dissected free and studied. The head was not used since the islets are less frequent in this part (*Warren et al.* 1966) and since the islets of the head regularly show a lesser degree of amyloidosis than those of other parts (Westermark, unpublished). The pancreases to be studied were freed from large vessels and from as much as possible of the connective tissue. A small piece was fixed in formalin, embedded in paraffin, sectioned and stained with alkaline Congo red for determination of the degree of amyloidosis. The rest of the pancreas was frozen and stored at –20° C until used. Only pancreases with relatively massive islet amyloidosis were used (Table 1).

Pancreases from young patients with no obvious islet amyloidosis were used as controls. Specimens of other tissues from patients with systemic amyloidosis of different types were taken for comparison of other amyloids with islet amyloid (Table 2).

Concentration of Amyloid

Each pancreas (weight 20–50 gm) was treated separately by a modification of the method described by *Pras et al.* (1968). The frozen tissue was sliced with a knife and homogenized in 0.15 M NaCl in a Buhler homogenizer. After centrifugation at 15,000 r.p.m. in a refrigerated Servall centrifuge, the sediment was rehomogenized in a Dounce homogenizer. This was repeated at least 10 times and was followed by homogenization in distilled water. After centrifugation, the pellets were frozen and cut into two pieces. The bottom layer, which contained most of the amyloid together with collagen, was saved and rehomogenized twice in distilled water. After centrifugation the pellets were frozen and again cut into two pieces

TABLE 2. *Patients with Systemic Amyloidosis*

Case No.	Sex	Age at death (years)	Major disease other than amyloidosis	Source of amyloid fibrils
582	F	46	Rheumatoid arthritis	Spleen
808	M	76	None	Spleen
758	M	54	Myelomatosis	Tongue

of which the bottom layer was saved and lyophilized.

Amyloid fibrils from tissues of patients with systemic amyloidosis were prepared by the method described by *Pras et al.* (1968).

Digestions

These were applied to only part of the pancreatic material. The lyophilized material was suspended in a 0.1 per cent solution of pepsin (1:3000, Kebo, Stockholm, Sweden) in 0.1 M HCl. The enzyme:substrate ratio was 1:10. After incubation at +37° C for about 24 hours the material was centrifuged and the sediment was washed twice with distilled water and lyophilized. This material was treated with collagenase (162 U/mg; P-L Biochemicals, Milwaukee, Wis, U.S.A.) as described by *Cohen & Calkins* (1964). The enzyme concentration and the enzyme:substrate ratio were the same as in the peptic digestion. Finally, the material was washed twice with distilled water and lyophilized. This material was used for most studies.

Solubility and Spectrophotometry

The solubility of different amyloid preparations in alkali was tested by agitation of 1.6 mg concentrated amyloid in 3 ml 0.1 M NaOH for 24 hours at room temperature. In some cases, the material was sonicated before agitation. After centrifugation, the protein concentrations were estimated by the Folin-Ciocalteaus reagent (*Bailey* 1967). The ultraviolet absorption spectra were determined on the NaOH dissolved materials described above and also on one islet and one systemic amyloid preparation which had been hydrolysed in 6 M HCl at 110° C for 20 hours in a N_2 atmosphere and, after evaporation, dissolved in 0.1 M NaOH. A Zeiss spectrophotometer was used in the range 220–340 nm.

Electrophoresis

Sodium dodecyl sulphate (SDS) electrophoresis was performed by the method of *Weber & Osborn* (1969) as modified by *Harada et al.* (1971). Either directly after the digestions or after defatting, about 3 mg of the native amyloid concentrates were dissolved in 6 M guanidine HCl buffered to pH 8.2 with 4.3 M tris-HCl containing 1 per cent mercaptoethanol. After dialysis and lyophilization, the material was dissolved in 1 per cent SDS with 0.1 per cent thioglycollic acid and 0.1 per cent dithiothreitol (DTT). In some cases, the material was dissolved directly in 1 per cent SDS with 0.1 per cent thioglycollic acid and sometimes with DTT; five per cent, 10 per cent or 15 per cent polyacrylamide gels were used and the electrophoresis was run with 6 mA/gel with bromphenol blue as migratory marker. The gels were stained with 0.25 per cent Coomassie blue in 7.5 per cent acetic acid and 5 per cent methanol for 2 hours and destained in 7.5 per cent acetic acid and 5 per cent methanol. Molecular weight determinations were performed on gels containing 15 per cent polyacrylamide. Calibrations were done with bovine serum albumin, horse heart cytochrome C, insulin and glucagon.

RESULTS

Homogenization of pancreatic tissue was rather difficult because of the large amounts of connective tissue in the glands of old persons. After a few manual homogenizations, the large connective tissue particles were removed and the rest of the material was easily homogenized. After repeated homogenizations, the sediment was investigated for amyloid. It was found that the amyloid of the

TABLE 3. *The Yield of Concentrated Islet Amyloid from Some Pancreases after the Various Steps*

Case No.	Pancreatic net weight (g)	Weight before peptic digestion (mg)	Weight before digestion with collagenase (mg)	Final weight (mg)
Is 42	40	500	113	58
Is 46	30	635	123	66
Is 74	37	550	58	a
Is 100	23	407	70	a
Is 105	44	663	166	a

a) Digestion by collagenase not performed.

islets invariably was to be found at the bottom of the sediment together with collagen. It was also found that the amyloid of the islets appeared as particles which varied in size and that the fibrils were not separated from each other as is seen in other amyloids. The digestions, especially by pepsin, reduced the amount of material considerably (Table 3). The amyloid did not seem to be affected, however. The amounts of concentrated amyloid obtained in this way were rather low, usually between 10 and 60 mg. The actual amounts of amyloid were still lower, of course, since the concentrates only contained 10–50 per cent of amyloid.

Solubility

The solubility of islet amyloid in alkali was low as compared with the systemic amyloids. As seen in Table 4, only small amounts of protein became dissolved in the islet amyloid preparations. However, the proportion of dissolved material increased significantly after sonication. After centrifugation, the sediment from the islet amyloid preparations was considerable. The solubility in other solvents was also low.

Spectrophotometry

Spectrophotometry of the concentrated amyloid revealed no obvious peaks (Fig. 1). The three preparations studied showed a slight irregularity between 280 and 290 nm, but not the typical tryptophane or tyrosine patterns. Sonication did not change the absorption spectrum. All the control amyloids had peaks at 280 and 289 nm, indicating the presence of tryptophane (Fig. 2). Acid hydrolysis altered the peak of the tested systemic amyloid (758) to 294 nm, which is typical of tyrosine, while the islet amyloid (Is 46) (Fig. 3) only showed a slight elevation in that area.

TABLE 4. *The Solubility of Different Amyloid Preparations in 0.1 M NaOH, Estimated by the Folin-Ciocalteau Reagent*

Amyloid No	Mg in solution per mg dry material: Without sonication	After sonication
808	0.47	ND*
582	0.38	ND
758	0.49	ND
Is 42	0.09	ND
Is 46	0.09	ND
Is 74	ND	0.21
Is 100	0.10	0.19
Is 105	0.05	0.18

* Not determined.

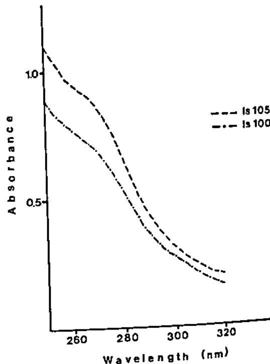


Fig. 1. Absorption spectra of two islet amyloids dissolved in 0.1 M NaOH. No obvious peaks indicating tryptophane or tyrosine are present.

SDS Electrophoresis

The electrophoresis of the non-sonicated pancreatic preparations revealed multiple weak bands prior to the digestions. After the peptic digestion most bands disappeared, but one or two weak bands persisted (Fig. 4). These bands moved as glucagon or a little slower, but in a few preparations a little more rapidly. Apart from the bands described, the gels of the islet amyloids only showed some slowly migrating, weak bands and differed

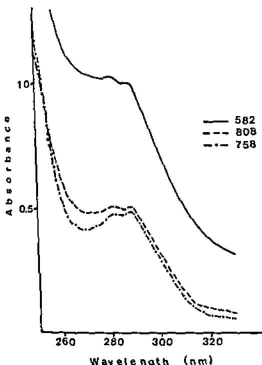


Fig. 2. Absorption spectra of three systemic amyloids dissolved in 0.1 M NaOH. Peaks typical of tryptophane are evident.

in that way from the control amyloids for which several distinct bands were seen.

After sonication, the bands only strengthened slightly and a broad zone without any distinct bands occurred in the range of 300,000–60,000 daltons; this was considered to be due to degradation of larger proteins. It was obvious that most of the material did not penetrate the gels, not even those containing 5 per cent polyacrylamide.

Control pancreas without islet amyloidosis displayed no rapidly moving bands before treatment with collagenase. Such treatment, however, resulted in a few bands which moved just a little slower than the bands of the islet amyloids.

DISCUSSION

When human amyloid rich tissues are homogenized and centrifuged, the amyloid fibrils usually form a top layer (*Cohen* & *Calkins* 1964). This is due to the separation of the single fibrils from each other. In contrast, the fragments of islet amyloid are constantly found in the bottom layer, mixed with collagen and other heavy particles. It has been possible to achieve further concentration of the impure sediment, since the islet amyloid seems to be as resistant to enzymes as are other amyloids (*Cohen* & *Calkins* 1964, *SriRam et al.* 1968).

The digestions with pepsin and collagenase thus reduced the material by up to 95 per cent. Electrophoresis showed a decrease of slowly migrating bands after the digestive processes but after digestion with collagenase there was a considerable increase of some rapidly migrating bands, probably due to decomposition of collagen. The final product contained up to 50 per cent of amyloid. Most of the non-amyloid consisted of thick collagen fibres, as revealed by electron microscopy.

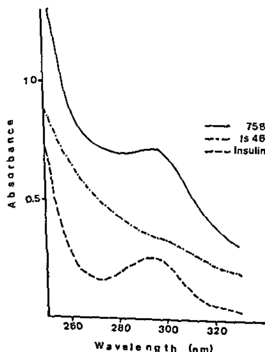


Fig. 3. Absorption spectra of one islet amyloid (Is 46), one systemic amyloid (758) and insulin after acid hydrolysis and solution in 0.1 M NaOH. Tryptophane is destroyed and the typical tyrosine peak at 294 nm is present in insulin and the systemic amyloid. The islet amyloid shows only a slight elevation in that area.

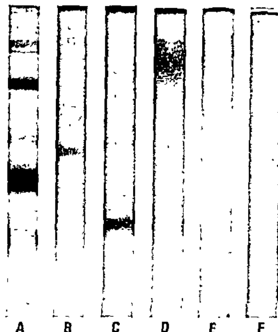


Fig. 4. SDS-polyacrylamide gel electrophoresis of bovine serum albumin, pepsin, cytochrome C, insulin and glucagon (A), primary systemic amyloid, case 808 (B), secondary systemic amyloid, case 582 (C), and islet amyloid, cases IS 100 (D), IS 105 (E) and Is 62 (F). The islet amyloids Is 100 and Is 105 were sonicated in 6 M guanidine HCl, while the islet amyloid Is 62 was only agitated in the same solution prior to electrophoresis.

The total yield of material from a single pancreas rarely exceeded 60 mg, i.e. maximally 30 mg of amyloid. Since the degree of amyloidosis of the islets usually is lower than 50 per cent (*Westermark* 1971), and as the islets constitute about 2 per cent of the tissue in the tail of the pancreas (*Westermark* & *Grimelius* 1973) and the dry weight of a pancreas used in this study usually was about 10 gm the yield can be estimated to be at least 20 per cent of the amyloid of a pancreas.

The reason why the islet amyloid is to be found in the bottom layer seems to be that the tufts of amyloid are held together very strongly. This phenomenon is probably one reason why it is so very difficult to dissolve the islet amyloid even in strongly denaturing agents such as 6 M guanidine hydrochloride. The effect of sonication on the solubility also supports this suggestion. However, differences in solubility of amyloid fibrils of different types are known to exist (*Benditt* & *Eriksen* 1971, *Harada et al.* 1971).

Electrophoresis of the crude islet amyloid material revealed weak bands, compared with the strong bands of other amyloids. The pattern of islet amyloid preparations from different individuals was usually constant, but slight differences did occur. Thus, it cannot be excluded that there are some inter-individual chemical dissimilarities in the islet amyloid. The strong correlation of islet amyloidosis to diabetes mellitus (*Bell* 1952, *Warren et al.* 1966 a.o.) and the morphological relationship between islet amyloid and β-cells (*Westermark* 1973a) indicate that the β-cells might be involved in the formation of the amyloid fibrils, and a relationship between islet amyloid and the normal function of the β-cells, i.e. synthesis and release of insulin, seems probable (*Westermark* 1973b). Indeed, under certain circumstances insulin can be transformed to long fibrils (*Waugh* 1946) which have properties in common with amyloid fibrils, e.g. green birefringence after Congo red staining (*Glenner et al.* 1974, *Westermark* 1974a). Islet amyloid treated for regeneration of insulin from fibrillar insulin (*Waugh* 1948) can yield insulin (IRI) (*Westermark* 1974a and b). The spectrophotometric pattern of islet amyloid, however, showed no or only a very slight elevation at 294 nm, typical of tyrosine (*Bailey* 1967), even after hydrolysis. Furthermore, the amino acid composition of the low molecular weight material corresponding to the fast moving bands on electrophoresis definitely differed from that of insulin, any of its chains, or C-peptide (*Westermark*, unpublished results). Therefore, C-peptide, insulin or ordinary insulin chains do not seem to constitute any major component of islet amyloid.

Insulin (IRI) can also be extracted from other amyloids, but, in contrast to islet amyloid, the extractable amounts do not increase on alkali treatment (*Westermark* 1974a and b). Why the IRI is more strongly

bound to islet amyloid remains to be explained.

Spectrophotometrically, islet amyloid lacks not only the tyrosine pattern but also significant amounts of tryptophane. This is in accordance with the histochemical findings (*Westermark* 1974a). Islet amyloids thus differs from most other amyloids studied (*Benditt* & *Eriksen* 1971, *Harada et al.* 1971). No peaks at 260 nm appeared in the spectrophotometer, indicating that nucleic acids, which are found in experimental murine amyloidosis (*Shapira et al.* 1973), constitute no major part of islet amyloid.

The electrophoretically fast moving bands of the amyloid-rich concentrates of the pancreases were the only ones which could be said to be of any appreciable strength, though they were never as strong as the major bands of the systemic amyloids or of amyloid of medullary carcinoma of the thyroid (*Westermark* 1975). However, most of the material did not enter the polyacrylamide gels and, furthermore, appeared as V_0 peak at gel filtration on a Sepharose 6 B column equilibrated with 5 M guanidine HCl (*Westermark*, to be published). In this latter respect, the islet amyloid and the amyloid of medullary carcinoma of the thyroid are similar (*Westermark* 1975). Furthermore, they both seem to have an epithelial origin (*Westermark* 1973a; *Ibanez* 1974) and contain no or only small amounts of tryptophane (*Pearse et al.* 1972, *Westermark* 1975). It is possible that the endocrine amyloid proteins constitute an entity, as has been proposed by *Pearse et al.* (1972). They would thus form a separate class of amyloid proteins to add to those already described (*Benditt* & *Eriksen* 1971, *Harada et al.* 1971).

Supported by grants from the *Swedish Medical Research Council* (Project No 102). Thanks are due to *Anni Bedy* and *Cristina Butt[illegible]ski* for skilled and generous assistance

REFERENCES

Bailey, J. L.: Techniques in protein chemistry. Amsterdam: Elsevier Publishing Company 1967.

Bell, E. T.: Hyalization of the islets of Langerhans in diabetes mellitus. Diabetes *1*: 341–344, 1952.

Benditt, E. P. & *Eriksen, N.*: Chemical classes of amyloid substance. Amer. J. Path. *65*: 231–250, 1971.

Benditt, E. P., Eriksen, N., Hermodson, M. A. & *Ericsson, L. H.*: The major proteins of human and monkey amyloid substance: common properties including unusual N-terminal amino acid sequences. Febs Letters *19*: 169–173, 1971.

Cohen, A. S. & *Calkins, E.*: The isolation of amyloid fibrils and a study of the effect of collagenase and hyaluronidase. J. Cell Biol. *21*: 481–486, 1964.

Ein, D., Kimura, S., Terry, W. D., Magnotta, J. & *Glenner, G. G.*: Amino acid sequence of an amyloid fibril protein of unknown origin. J. Biol. Chem. *247*: 5653–5655, 1972.

Glenner, G. G., Cuatrecasas, P., Isersky, C., Bladen, H. A. & *Eanes, E. D.*: Physical and chemical properties of amyloid fibres. II. Isolation of a unique protein constituting the major component from human splenic amyloid fibril concentrates. J. Histochem. Cytochem. *17*: 769–780, 1969.

Glenner, G. G., Harada, M., Isersky, C., Cuatrecasas, P., Page, D. & *Keiser, H.*: Human amyloid protein: Diversity and uniformity. Biochem. Biophys. Res. Commun. *41*: 1013–1019, 1970a.

Glenner, G G., Harbaugh, J., Ohms, J. I., Harada, M. & *Cuatrecasas, P.*: An amyloid protein: the amino-terminal variable fragment of an immunoglobulin light chain. Biochem. Biophys. Res. Commun. *41*: 1287–1289, 1970b.

Glenner, G. G., Terry, W., Harada, M., Isersky, C. & *Page, D.*: Amyloid fibril proteins: Proof of homology with immunoglobulin light chains by sequence analysis. Science *172*: 1150–1151, 1971.

Glenner, G. G., Harada, M. & *Isersky, C.*: The purification of amyloid fibril proteins. Prep. Biochem. *2*: 39–51, 1972.

Glenner, G. G., Eanes, E. D., Bladen, H. A., Linke, R. P. & *Termine, J. D.*: β-pleated sheet fibrils. A comparison of native amyloid with synthetic protein fibrils. J. Histochem. Cytochem. *22*: 1141–1158, 1974.

Harada, M., Isersky, C., Cuatrecasas, P., Page, D., Bladen, H. A., Eanes, E. D., Keiser, H. R. & *Glenner, G. G.*: Human amyloid protein: chemical variability and homogeneity. J. Histochem. Cytochem. *19*: 1–15, 1971.

Husby, G., Sletten, K., Michaelsen, T. E & *Natvig, J. B.*: Alternative, non-immunoglobulin origin of amyloid fibrils. Nature New Biology *238*: 187–188, 1972.

Husby, G., Natvig, J. B., Michaelsen, T. E., Sletten, K. & *Höst, H.*: Unique amyloid protei

subunit common to different types of amyloid fibril. Nature *244:* 362–264, 1973a.

Husby, G., Sletten, K., Michaelsen, T. E. & Natvig, J. B.: Amyloid fibril protein subunit, "Protein AS": distribution in tissue and serum in different clinical types of amyloidosis including that associated with myelomatosis and Waldenström's macro-globulinaemia. Scand. J. Immunol. *2:* 395–404, 1973b.

Husby, G., Natvig, J. B. & Sletten, K.: New, third class of amyloid fibril protein. J. Exp. Med. *139:* 773–778, 1974.

Ibanez, M. L.: Medullary carcinoma of the thyroid gland. In: Pathology Annual, Sommers, S. C., ed. p. 263: Appleton-Century-Crofts, 1974.

Levin, M., Franklin, E. C., Frangione, B. & Pras, M.: The amino acid sequence of a major nonimmunoglobulin component of some amyloid fibrils. J. Clin. Invest. *51:* 2773–2776, 1972.

Opie, E. L.: On relation of chronic interstitial pancreatitis to the islands of Langerhans and to diabetes mellitus. J. Exp. Med. *5:* 397–428, 1900.

Pearse, A. G. E., Ewen, S. W. B. & Polak, J. M.: The genesis of apud amyloid in endocrine polypeptide tumours: histochemical distinction from immun amyloid. Virchows Arch. Abt. B. *10:* 93–107, 1972.

Pras, M. & Reshef, T.: The acid-soluble fraction of amyloid—a fibril forming protein. Biochim. Biophys. Acta *271:* 193–203, 1972.

Pras, M., Schubert, M., Zucker-Franklin, D., Rimon, A. & Franklin, E. C.: The characterization of soluble amyloid prepared in water. J. Clin. Invest. *47:* 924–933, 1968.

Pras, M., Zucker-Franklin, D., Rimon, A. & Franklin, E. C.: Physical, chemical and ultrastructural studies of water-soluble human amyloid fibrils. Comparative analyses of nine amyloid preparations. J. Exp. Med. *130:* 777–795, 1969.

Shapira, E. & Farhi, R.: Comparison of some murine and human amyloid preparations Br. J exp Path. *54* 518–523, 1973.

Sletten, K., Husby, G. & Natvig, J. B.: N-terminal amino acid sequence of amyloid fibril protein AR, prototype of a new λ-variable subgroup, VλV. Scand. J. Immunol. *3:* 833–836, 1974

SriRam, J., DeLellis, R. A. & Glenner, G. G.: Amyloid IV. Is human amyloid immunogenic? Int. Arch. Allergy *34:* 269–282, 1968.

Warren, S., LeCompte, P. M. & Legg, M. A.: The pathology of diabetes mellitus. 4th ed. Philadelphia: Lea and Febiger 1966.

Waugh, D. F.: A fibrous modification of insulin. I. The heat precipitate of insulin. J. Amer. chem. Soc. *68:* 247–250, 1946.

Waugh, D. F.: Regeneration of insulin from insulin fibrils by the action of alkali. J. Amer. chem. Soc. *70:* 1850–1857, 1948.

Weber, K. & Osborn, M.: The reliability of molecular weight determinations by dodecyl sulphate-polyacrylamide gel electrophoresis. J. Biol. Chem. *244:* 4406–4412, 1969.

Westermark, P.: Mast cells in the islets of Langerhans in insular amyloidosis. Virchows Arch. Abt. A. *354.* 17–23, 1971.

Westermark, P.: Fine structure of islets of Langerhans in insular amyloidosis. Virchows Arch. Abt. A. *359:* 1–18, 1973a.

Westermark, P.: Amyloidosis in the islets of Langerhans in man. Thesis, Uppsala, 1973b.

Westermark, P.: On the nature of the amyloid in human islets of Langerhans. Histochemistry *38:* 27–33, 1974a.

Westermark, P.: Islet amyloid—a fibrillar form of insulin? Acta endocrinol. *77:* suppl. *190:* 35, 1974b.

Westermark, P.: Amyloid of medullary carcinoma of the thyroid: Partial characterization. Uppsala J. Med Sci. *80.* 88–92, 1975.

Westermark, P. & Grimelius, L.: The pancreatic islet cells in insular amyloidosis in human diabetic and non-diabetic adults. Acta path. microbiol. scand. Sect. A, *81.* 291–300, 1973.

Westermark, P., Skinner, M. & Cohen, A. S.: The P-component of amyloid of human islets of Langerhans. Scand. J Immunol. *4:* 95–97, 1975.

Acta path. microbiol. scand. Sect. C, 83: 447–454, 1975

DRUG EFFECTS ON HUMAN LEUCOCYTE MIGRATION AND MIGRATION INHIBITORY ACTIVITY FROM LYMPHOCYTES STIMULATED WITH CONCANAVALIN A

KLAUS BENDTZEN

Laboratory of Clinical Immunology, Medical Department TA,
Rigshospitalet University Hospital, Copenhagen, Denmark

Bendtzen, K. Drug effects on human leucocyte migration and migration inhibitory activity from lymphocytes stimulated with concanavalin A. Acta path. microbiol. scand. Sect. C, 83: 447–454, 1975.

The *in vitro* effect of a number of drugs on human peripheral blood leucocyte migration and migration inhibitory factors produced by lymphocytes stimulated with concanavalin A was studied Using the indirect leucocyte migration agarose technique, migration was not influenced by cyclophosphamide, diethylstilbestrol or puromycin. Theophylline (2 μg/ml) marginally inhibited, and methylprednisolone (2.5 μg/ml) markedly stimulated, cell migration under agarose. Production of leucocyte migration inhibitory activity (LMIA) was inhibited by theophylline (20 and 200 μg/ml) and abolished by methylprednisolone (2.5 μg/ml), cyclophosphamide (2.5 and 25 μg/ml), diethylstilbestrol (0.01 μg/ml) and puromycin (0.1 to 10 μg/ml). The influence of LMIA on migrating cells was inhibited by puromycin (1 and 10 μg/ml) and by high concentrations of diethylstilbestrol (0.1 μg/ml).

Key words: Leucocyte migration; migration inhibitory factor; drug effects.

K. Bendtzen, Laboratory of Clinical Immunology, Medical Department TA, Rigshospitalet University Hospital, Tagensvej 18, DK-2200 Copenhagen N, Denmark.

Received 29.vii 75 Accepted 29.vii 75

Sensitized lymphocytes incubated with the specific antigen as well as normal lymphocytes stimulated with mitogens generate substances which inhibit the migration of guinea pig peritoneal macrophages and human peripheral blood leucocytes *in vitro* (4, 9). The lymphocyte product with such an activity is known as migration inhibitory factor (MIF) although it seems more appropriate to speak about a number of inhibitory factors (23). The mechanisms by which these factors are generated from lymphocytes and cause inhibition of migration are unknown, but the two steps of the process seem independent of each other. In the past, migration inhibition tests designed to elucidate the *in vitro* effect of various agents on cell-mediated hypersensitivity have been difficult to interpret since effects on MIF production and MIF action were not separated. The present paper describes a two-step technique for examining both the production by lymphocytes of factors with leucocyte migration inhibitory activity (LMIA) and the LMIA-leucocyte interaction. The technique provides opportunity

to obtain information about effects of chemicals and drugs on these processes, and the modifying influence of methylprednisolone, *cyclophosphamide*, *diethylstilbestrol*, *theo*phylline and puromycin is reported.

MATERIALS AND METHODS

Lymphocyte Culture

Venous blood from healthy human adults of both sexes was collected and the mononuclear cells were separated by centrifugation in an Isopaque-Ficoll gradient (Lymphoprep®, Nyegaard & Co, *Oslo, Norway*). *The cells were then washed three* times in Hanks' solution and suspended in serum-free medium TC-199 with penicillin 67 i.u. and streptomycin 67 μg/ml (Difco Laboratories, Michigan, U.S.A.) (TC-199).

Production of LMIA

3 ml cell suspensions (2.5 × 10^6 cells/ml) were incubated for 22 h with 250 μg concanavalin A (Pharmacia, Uppsala, Sweden) (Con A) at 37° C in 2 per cent CO_2 in air saturated with water vapor. Control cultures grown without Con A were reconstituted with the same amount of mitogen. Supernatants were subsequently applied *on small* columns of Sephadex G-100 (Pharmacia) and eluted with TC-199 as previously described (4). Thus, all detectable Con A was removed by conjugation to the Sephadex. After passage the original *supernatants were diluted 1:3. Eluates were* passed through Millipore filters (0.45 μm pore size), tested immediately or kept at –20° C.

Assay for LMIA

The indirect leucocyte migration agarose technique described by *Clausen* (7) was employed using as migratory cells peripheral blood leucocytes *from healthy, unrelated adults.* 22 × 10^6 *cells were* suspended in 90 μl culture supernatant and tested for migration under agarose. The LMIA was determined as a migration index·

$$MI = \frac{\text{Mean area of migration, using Con A-stimulated supernatants}}{\text{Mean area of migration, using parallel control supernatants}}$$

Effect of Drugs on Leucocyte Migration

The same procedure as that described above was followed. 22 × 10^6 cells were suspended in 90 μl TC-199 (control leucocytes) and 90 μl TC-199 containing the drug tested ("treated leucocytes") The migration index was calculated using the formula:

$$MI = \frac{\text{Mean area of migration of "treated leucocytes"}}{\text{Mean area of migration of control leucocytes}}$$

Effect of Drugs on LMIA Production

The drugs were added both to Con A-preincubated and to unstimulated control cell suspensions and incubated for 22 h. Control cultures were reconstituted with Con A and all supernatants *were* passed through Sephadex G-100. *In order to* remove the drugs, the eluates were dialyzed against TC-199 for 20 h before assay for LMIA.

Effect of Drugs on LMIA-leucocyte Interaction

The agents tested were added both to LMIA-*containing supernatants and control supernatants* after removal of mitogen on Sephadex. Supernatants were then assayed for LMIA.

The effects of drugs on LMIA-production and -leucocyte interaction were calculated by comparing *the MI in the presence of the drug* (MI_{Drug}) with that in the absence of the drug ($MI_{Control}$):

$$\text{Per cent inhibition} = \frac{MI_{Drug} - MI_{Control}}{1 - MI_{Control}} \times 100$$

All reported values represent means of four experiments using different cell *populations* Each experiment was set up in quadruplicate. The significance of effect of a drug was calculated using the Mann-Whitney rank sum test

Drugs and Chemicals

Methylprednisolone acetate (*Hoechst, Frankfurt* a Main, Germany), diethylstilbestrol phosphate (Honvan®, Asta-Werke A.G., Westfalen, Germany) *and theophyllaminum*, a combination of ethylenediamine and theophylline 20 mg/ml (Dispensary, Rigshospitalet, *Copenhagen, Denmark*) *were* obtained as sterile solutions and diluted in TC-199. Cyclophosphamide (Endoxan®, Asta-*Werke*) *and* puromycin dihydrochloride (Sigma, St. Louis, Mo, U.S.A.) were dissolved in TC-199. All chemicals *were stored at* –20° C until used.

RESULTS

The different concentrations of drugs tested and the clinical relevance are shown in Table 1.

A. Effect of Methylprednisolone

In vitro migration of leucocytes were significantly enhanced by the drug at a concen-

TABLE 1. *The Concentrations of Drugs Compared with Average Physiological and Therapeutic Plasma Levels*

Drug	Concentrations used in the experiments (μg/ml)	Average physiological plasma concentrations (10) (μg/ml)	Average therapeutic plasma concentrations (μg/ml)
Methylprednisolone (MP)	0.025 0.25 2.5	0.1 cortisol (=) 0.025 MP*	0 025 to 0.25 (2.5)
Cyclophosphamide	0 25 2.5 25	–	Rapidly metabolized in the liver
Diethylstilbestrol (DS)	0.0001 0.001 0 01 0.1	<0.002 estrone (=) DS Late pregnancy: 0.1 estrone (=) DS	0.5 (cancer chemotherapy)
Theophylline	2 20 200	–	20
Puromycin	0.1 1 10	–	Not clinically used

* with respect to antiinflammatory activity.

TABLE 2. *Effect of Drugs on Human Peripheral Blood Leucocyte Migration*

Drug	Concentration (μg/ml)	MI ± SEM*	P
Methylprednisolone	0.025 0.25 2.5	1.07 ± 0.14 1.11 ± 0.09 1.39 ± 0.11	ns† ns <0 05
Cyclophosphamide	0 25 2.5 25	0.94 ± 0.04 0.95 ± 0.05 0.97 ± 0.06	ns ns ns
Diethylstilbestrol	0 001 0 01 0.1	1.09 ± 0.10 1.02 ± 0.06 0.99 ± 0.03	ns ns ns
Theophylline	2 20 200	0.90 ± 0.03 0.95 ± 0.06 1.29 ± 0.14	<0.05 ns ns
Puromycin	0.1 1 10	1.06 ± 0.05 1.03 ± 0.06 1.00 ± 0.07	ns ns ns

* Migration index ± standard error of the mean.
† P>0.05 was considered not significant (Mann-Whitney rank sum test).

TABLE 3. *Effect of Drugs on LMIA* Production*

LMIA without drug MI ± SEM†	Drug	Concentration (μg/ml)	Inhibition of LMIA production (per cent)	P
		0.025	39	<0.05
0.77 ± 0.02	*Methylprednisolone*	0.25	52	<0.05
		2.5	100	<0.05
		0.25	79	ns§
0.76 ± 0.02	Cyclophosphamide	2.5	100	<0.05
		25	100	<0.05
		0.0001	28	ns
0.68 ± 0.08	Diethylstilbestrol	0.001	78	ns
		0.01	100	<0.05
		2	55	*ns*
0.69 ± 0.08	Theophylline	20	87	<0.05
		200	81	<0.05
		0.1	100	<0.05
0.74 ± 0.01	Puromycin	1	88	<0.05
		10	100	<0.05

* *Leucocyte migration inhibitory activity produced by human lymphocytes stimulated* with concanavalin A.
† Migration index ± standard error of the mean.
§ *P>0.05 was considered not significant* (Mann-Whitney *rank sum test*).

tration of 2.5 μg/ml (Table 2). At the same concentration, the LMIA production was completely abolished, the effect being detectable even *in the low* dose tested (Table 3). No significant effect on LMIA-leucocyte interaction could be found (Table 4).

B. Effect of Cyclophosphamide

A slight but insignificant reduction in leucocyte migration was detected, the effect almost disappearing in the highest dose tested (Table 2). The action on LMIA-production and -target cell interaction appeared to be the same as that of methylprednisolone (Table 3 and 4).

C. Effect of Diethylstilbestrol

This synthetic compound with a relative estrogenic strength as estrone did not significantly *influence* the *leucocyte migration* at concentrations of 0.001 to 0.1 μg/ml (Table 2). On the other hand, LMIA-leucocyte interaction and particularly LMIA *production* were inhibited in concentrations of 0.01 and 0.1 μg/ml (Table 3 and 4).

D. Effect of Theophylline

At a concentration of 2 μg/ml, the drug consistently *inhibited the leucocyte* migration, but the inhibition, though statistically significant, was only marginal (Table 2). *The stimulatory* activity of 200 μg/ml was not significant. The drug had no activity on LMIA-*leucocyte interaction*, but the LMIA production was significantly inhibited at concentrations of 20 and 200 μg/ml (Table 3).

E. Effect of Puromycin

The drug did not affect leucocyte migration even in the highest concentration which has been found to inhibit the capillary tube *migration of guinea pig* macrophages (21) (Table 2). LMIA production was consistently inhibited at *all concentrations* tested,

TABLE 4. *Effect of Drugs on LMIA* Action*

LMIA without drug MI ± SEM†	Drug	Concentration (μg/ml)	Inhibition of LMIA action (per cent)	P
0.59 ± 0.06	Methylprednisolone	0.025	12	ns§
		0.25	15	ns
		2.5	20	ns
0.65 ± 0.09	Cyclophosphamide	0.25	34	ns
		2.5	40	ns
		25	31	ns
0.72 ± 0.04	Diethylstilbestrol	0.001	36	ns
		0.01	50	ns
		0.1	64	<0.05
0.79 ± 0.02	Theophylline	2	no	–
		20	no	–
		200	24	ns
0.65 ± 0.03	Puromycin	0.1	23	ns
		1	71	<0.05
		10	63	<0.05

* Leucocyte migration inhibitory activity produced by human lymphocytes stimulated with concanavalin A.
† Migration index ± standard error of the mean.
§ P>0.05 was considered not significant (Mann-Whitney rank sum test).

and at concentrations of 1 and 10 μg/ml the agent counteracted LMIA-leucocyte interaction as well (Table 3 and 4).

To rule out the possibility that Con A itself reacted with the drugs, Con A was incubated at 37° C for 60 min in the presence or absence of the agents. These were then removed on TC-199-equilibrated columns of Sephadex G-25. Since Sephadex G-25 cannot bind Con A (2), active Con A or Con A inactivated by the drug would appear in the eluate. In tests of supernatants from lymphocytes stimulated with eluates containing Con A or drug-pre-incubated Con A, the LMIA's were always found to be identical.

DISCUSSION

The study describes the application of an *in vitro* technique for testing drug influence on selected parts of the effector mechanism in cell-mediated hypersensitivity. The leucocyte migration as well as the mitogen-induced LMIA production and the LMIA-induced inhibition of leucocyte migration seem to be extremely sensitive to a number of drugs and chemicals.

Upon specific stimulation, lymphocytes have been found to release several biologically active substances commonly known as lymphokines (11). As Con A induces lymphocyte transformation (25) and lymphokine production (4) in the absence of serum, this substance was chosen as lymphocyte activator. Thus, the protein binding of drugs was greatly minimized.

The necessary removal of biologically active traces of Con A before measuring the LMIA was efficiently accomplished by passage of the supernatants through Sephadex G-100 columns, which have sufficiently high absorption affinity for Con A. Since Con A causes migration inhibition of leucocyte cultures, this important technical point was carefully checked in each case by parallel control experiments using comparable, Con A-

containing, lymphocyte-free supernatants (4).

As almost any compound at a certain high concentration influences leucocyte migration because of unspecific toxicity, it was essential to test the drugs only at concentrations which did not in themselves cause inhibition of cell migration. One exception to this was in this study the inclusion of theophylline 2 μg/ml. At this rather low concentration a slight inhibition could be demonstrated which, however, disappeared at higher concentrations.

Methylprednisolone

The mechanism by which glucocorticoids suppress immunological functions is on the whole unknown despite many years of empirical use of these agents. Significant species differences exist *in vivo* and *in vitro* and must be borne in mind when results obtained by different animal test systems are to be compared. Thus, *Balow* & *Rosenthal* (3) who used guinea pigs reported that tuberculin (PPD)-induced MIF activity would be inhibited in a dose-related fashion by hydrocortisone in doses of 1 to 100 μg/ml. At a concentration of 10 μg/ml, the drug did not affect the production of MIF by immune lymphocytes. Unfortunately, the effect on macrophage migration was not determined. These findings are inconsistent with those described in the present paper according to which LMIA production in man is inhibited. However, also the experimental assays are different, thereby impeding conclusive comparison. In man, glucocorticoids also suppress antigen- and phytohaemagglutinin (PHA)-induced lymphocyte transformation and the mixed lymphocyte reaction (13, 14, 19).

Cyclophosphamide

This immunosuppressive, *in vivo*, requires metabolic activation in the hepatic microsomal enzyme system (12). Even so, a profound *in vitro* effect of the non-activated drug could be demonstrated. Since cyclophosphamide depletes lymphocytes from the non-thymus-dependent areas of the lymph nodes and spleen, leaving the thymus-dependent areas essentially intact (24) and increases the proportion of theta-positive lymphocytes in these organs (22), the immunosuppressive effect might be due to elimination of the B-cell response. However, the inhibition of LMIA production seems to involve T-cell functions as well, although the concept of soluble Con A as a pure T-cell mitogen recently (6) has been challenged.

Diethylstilbestrol

In non-pathological conditions, the high concentrations of estrogenic activity by which LMIA production is inhibited is seen only during late stages of pregnancy and is then due to placental secretion (10). This may well be one of the mechanisms by which the foetus avoids allograft rejection. PHA-induced lymphocyte transformation is suppressed by the very high concentrations of diethylstilbestrol to be achieved in the treatment of patients with hormonally responsive tumours (1). No effect on leucocyte migration was detected although estrogens have been shown to stimulate macrophage activity (18).

Theophylline

This xanthine derivative is capable of raising the endogenous level of cyclic 3', 5'-AMP by competitive inhibition of nucleotide phosphodiesterase, the cyclic AMP degrading enzyme (5). *Pick* & *Manheimer* (21) found potent inhibition of guinea pig macrophage migration at theophylline concentrations of 20 and 200 μg/ml. In contrast to this, findings by *Koopman et al.* (15) and findings in the present study show no effect of similar theophylline concentrations on guinea pig macrophage and human leucocyte migration, respectively. Agents known to increase cyclic AMP levels in a variety of tissues have been shown to prevent antigen-induced (21) and mitogen-induced (15) inhibition of migration of guinea pig macrophages. Whether or not an effect was due to inhibition of MIF

production was not determined. It was observed in the present study that, in concentrations of 20 and 200 $\mu g/ml$, the drug inhibited the LMIA production, but had no effect on LMIA-leucocyte interaction. This is in agreement with reports according to which MIF production is reduced by guinea pig lymph node cells stimulated with PPD (20). While the methylxanthines are of considerable importance with respect to cyclic AMP action, other effects of these compounds have been demonstrated. As an example theophylline reduces the binding of calcium in membranes which may play a role in lymphokine production and action. Thus, removal of calcium from culture medium enhances macrophage migration and abolishes MIF activity (21).

Puromycin

Puromycin has been shown to suppress protein synthesis by effecting premature release of partially formed peptides from ribosomes (17) and inhibiting the incorporation of ^{14}C-leucine into the peritoneal exudate cell protein (8). Many workers agree that puromycin, if present in the media at a nontoxic concentration of 10 $\mu g/ml$ is capable of blocking the antigen-induced migration inhibition of sensitive cells (8, 16, 21). The profound effect of puromycin, even at very low concentrations, on LMIA production speaks in favour of the hypothesis that LMIA is actively synthetized by the lymphocytes after mitogenic stimulation. However, multiple explanations of the mechanism are possible since widespread changes in cell function and metabolism must follow the suppression of protein synthesis.

This study and further similar experiments should prove useful in two ways. Firstly, a description of enhancing and inhibiting effects of relevant chemicals on cell migration and their modifying influence on LMIA should be helpful in elucidating *in vitro* expressions of cell-mediated hypersensitivity. Secondly, an *in vitro* technique for testing drug effects on the efferent cell-mediated immune response is needed in the efforts to find new immunoregulatory and selective antiinflammatory agents for clinical use.

The study was supported by grants from the *Danish Hospital Foundation for Medical Research*, Region of Copenhagen, Greenland and the Faroe Islands, and the *Danish Medical Research Council*.

REFERENCES

1. *Ablin, R. J., Bruns, G. R., Guinan, P. & Bush, I. M.:* The effect of estrogen on the incorporation of ^{3}H-thymidine by PHA-stimulated human peripheral blood lymphocytes. J. Immunol. *113:* 705–707, 1974.
2. *Agrawal, B. B. L. & Goldstein, I. J.:* Protein-carbohydrate interaction. VI. Isolation of concanavalin A by specific adsorption on cross-linked dextran gels. Biochim. Biophys. Acta *147:* 262–271, 1967.
3. *Balow, J. E. & Rosenthal, A. S.:* Glucocorticoid suppression of macrophage migration inhibitory factor. J. Exp. Med. *137:* 1031–1041, 1973.
4. *Bendtzen, K., Andersen, V. & Bendixen, G.:* An *in vitro* assay of leukocyte migration inhibitory activity (LMIA) from human lymphocytes stimulated with concanavalin A. Acta allergol. *30:* 133–149, 1975.
5. *Butcher, R. W. & Sutherland, E. W.:* Adenosine 3', 5'-phosphate in biological materials. I. Purification and properties of cyclic 3', 5'-nucleotide phosphodiesterase and the use of this enzyme to characterize adenosine 3', 5'-phosphate in human urine. J. biol. Chem. *237:* 1244–1250, 1962.
6. *Chess, L., MacDermott, R. P. & Schlossman, S. F.:* Immunologic functions of isolated human lymphocyte subpopulations. I. Quantitative isolation of human T and B cells and response to mitogens. J. Immunol. *113:* 1113–1121, 1974.
7. *Clausen, J. E.:* Migration inhibitory effect of cell-free supernatants from mixed human lymphocyte cultures. J. Immunol. *108*, 453–459, 1972.
8. *David, J. R.:* Suppression of delayed hypersensitivity in vitro by inhibition of protein synthesis. J. Exp. Med. *122:* 1125–1134, 1965.
9. *David, J. R., Al-Askari, S., Lawrence, H. S. & Thomas, L.:* Delayed hypersensitivity in vitro. I. The specificity of inhibition of cell migration by antigens. J. Immunol. *93*, 264–273, 1964.
10. *Diem, K.* (Edit.): Documenta Geigy Scientific Tables. J. R. Geigy S.A. ...

11. *Dumonde, D. C., Wolstencroft, R. A., Panayi, G. S., Matthew, M., Morley, J. & Howson, W. T.:* "Lymphokines": Non-antibody mediators of cellular immunity generated by lymphocyte activation. Nature *224:* 38–42, 1969.
12. *Goodman, L. S. & Gilman, A.* (Edit.): The *Pharmacological Basis of* Therapeutics. The Macmillan Company, New York 1970.
13. *Heilman, D. H., Gambrill, M. R. & Leichner, J. P.:* The effect of hydrocortisone on the incorporation of tritiated thymidine by human blood lymphocytes cultured with phytohaemagglutinin and pokeweed mitogen. Clin. exp. Immunol. *15:* 203–212, 1973.
14. *Heilman, D. H. & Leichner, J. P.:* The effect of cortisol on the transformation of human blood lymphocytes by antigens and allogeneic leukocytes. In: Schwartz, M. R. (Ed.): Proceedings of the sixth leucocyte culture conference, Academic Press, New York 1971, p. 581.
15. *Koopman, W. J., Gillis, M. H. & David, J. R.:* Prevention of MIF activity by agents known to increase cellular cyclic AMP. J. Immunol. *110:* 1609–1614, 1973.
16. *Mitchell, C. G., Smith, M. G. M., Golding, P. L., Eddleston, A. L. W. F. & Williams, R.:* Evaluation of the leucocyte migration test as a measure of delayed hypersensitivity in man. *Suppression of migration inhibition by puromycin.* Clin. exp. Immunol. *11:* 535–541, 1972.
17. *Nathans, D.:* Inhibition of protein synthesis by puromycin. Fed. Proc. *23:* 984–989, 1964.
18. *Nicol, T., Bilbey, D. L. J., Charles, L. M., Cordingley, J. L. & Vernon-Roberts, B.:* Oestrogen: The natural stimulant of body defence. J. Endocrinol. *30:* 277–291, 1964.
19. *Nowell, P. C.:* Inhibition of human leukocyte *mitosis by prednisolone in vitro.* Cancer Res. *21:* 1518–1521, 1961.
20. *Pick, E.:* Soluble lymphocytic mediators. I. Inhibition of macrophage migration inhibitory factor production by drugs. Immunology *26:* 649–658, 1974.
21. *Pick, E. & Manheimer, S.:* The mechanism of action of soluble lymphocytic mediators. II. Modification of macrophage migration and *migration inhibitory factor action by drugs, enzymes and cationic environment.* Cell. Immunol. *11:* 30–46, 1974.
22. *Poulter, L. W. & Turk, J. L.:* Proportional increase in the θ carrying lymphocytes in *peripheral lymphoid tissue following* treatment with cyclophosphamide Nature New Biol. *238:* 17–18, 1972.
23. *Rocklin, R. E.:* Products of activated lymphocytes: Leukocyte *inhibitory factor (LIF) distinct from migration* inhibitory factor (MIF). J. Immunol. *112:* 1461–1466, 1974.
24. *Turk, J. L. & Poulter, L. W:* Selective depletion of lymphoid tissue by cyclophosphamide. *Clin. exp. Immunol. 10.* 285–296, 1972.
25. *Williams, D. R. R. & Coulson, A S.:* Lymphocyte transformation in protein-free medium by concanavalin A. Lancet *i:* 80, 1971.

Acta path microbiol. scand. Sect. C, *83:* 455–458, 1975

DETERMINATION OF HUMAN ANTIBODIES TO STAPHYLOCOCCAL α-TOXIN (ANTISTAPHYLOLYSIN) BY A REVERSED SINGLE RADIAL IMMUNODIFFUSION METHOD

Ulf Johnson and Birgitta Svantesson

Institute of Medical Microbiology, Department of Immunology, University of Lund, Lund, Sweden

Johnson, U. & Svantesson, B. Determination of human antibodies to staphylococcal α-toxin (antistaphylolysin) by a reversed single radial immunodiffusion method Acta path. microbiol. scand. Sect. C, *83:* 455–458, 1975.

A method for the routine determination of human antibodies to staphylococcal α-toxin (antistaphylolysin, ASTA), performed as a reversed single radial immunodiffusion, is presented. The sera to be tested are allowed to diffuse into a staphylolysin-containing gel. A second gel containing rabbit erythrocytes is poured on top of the first gel and, after diffusion of free staphylolysin, the zones of inhibited hemolysis are measured. The method was compared with the conventional tube-dilution method and the correlation coefficient (r) was found to be 0.95.

Key words: Staphylococcal α-toxin; antibodies; radial immunodiffusion.

Ulf Johnson, Institute of Medical Microbiology, Sölvegatan 23, S-223 62 Lund, Sweden.

Received 12.vii.75 Accepted 5.viii.75

Antibodies to staphylococcal α-toxin (ASTA) in blood from healthy individuals as well as from those infected with staphylococci were demonstrated as early as 1901 by *Neisser & Wechsberg* (1901). ASTA determinations are usually performed as a tube-dilution test originally described by *Neisser & Wechsberg* (1901) in which a standardized dose of staphylococcal α-toxin is added to the serum under test and this mixture is assayed for hemolysis of rabbit erythrocytes.

Zetterall (1968) described a micro-method for determining ASTA based on gel diffusion. The ASTA-containing material was allowed to diffuse into a gel containing rabbit erythrocytes, after which staphylococcal α-toxin was diffused into the system. Zones of inhibited hemolysis were measured.

In 1974 *Brorson et al.* (1974) presented a gel-diffusion method for determining ASTA titres below 2 U/ml. Sera containing ASTA above 2 U/ml had to be titrated by the conventional tube-dilution method.

This paper describes a simple, accurate and inexpensive micro-method for the routine estimation of ASTA based on reversed single radial immunodiffusion.

MATERIALS

.bit erythrocytes were washed 3 times in 0.15 M NaCl and a 2 per cent suspension in 0.15 M NaCl was prepared.

Standard preparation of staphylolysin (α-toxin), N:o 36, 20 U/ml, was obtained from the Swedish State Bacteriological Laboratory, Solna, Stockholm, Sweden.

Standard preparation of staphylococcal antitoxin (anti-α-toxin), 20 U/ml, was obtained from the World Health Organization, International Laboratory for Biological Standards, Statens Seruminstitut, Copenhagen, Denmark.

Agarose (Miles Seravac Ldt, Maidenhead, Great Britain) was dissolved in phosphate buffered saline 0.15 M, pH 7.2 (PBS).

RESULTS

A solution of 0.6 per cent (w/v) agarose in PBS was prepared. After having cooled to 50° C staphylolysin (20 U/ml) was added to a final concentration of 1 per cent. Care must be taken to avoid temperatures above 50° C as the toxin gradually lost activity at higher temperatures. The mixture was poured into a mould made of two thin glass plates (1 × 205 × 110 mm) separated by a U-shaped

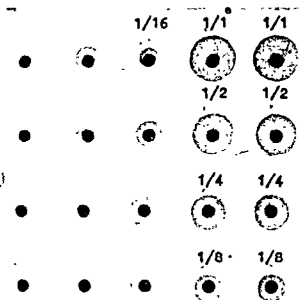


Fig. 1. The ASTA-content in 15 sera estimated with th twice. As reference a double serial dilution of a stand U/ml) is used.

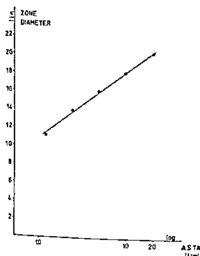


Fig. 2. The standard curve constructed by plotting the sum of two perpendicular zonediameters against the ASTA-content. A serial dilution containing 20, 10, 5, 2.5 and 1.25 U/ml was used

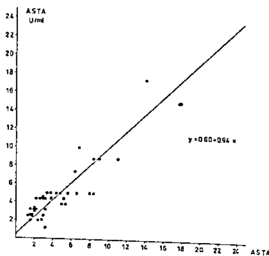


Fig. 3. Correlation between the gel-diffusion method (x-axis) and the tube-dilution method (y-axis). The ASTA content of 41 sera with elevated ASTA titres were analysed. The correlation coefficient (r) was 0.95 and the regression line y = 0.60 + 0.94×.

agarose solution in PBS with 10 ml of a 2 per cent suspension of rabbit erythrocytes. The mixture was rapidly warmed to 45° C in a waterbath and then poured over the first gel, which was placed on a horizontal table and surrounded by a 4-sided perspex frame 4 mm thick. The plate was incubated in a moist chamber at 37° C to allow free staphylolysin to diffuse into the erythrocyte containing gel. After 30 minutes circular zones of inhibited hemolysis appeared around the holes (Fig. 1).

The plate was covered with a filter paper soaked in PBS and on top of this a 10 mm layer of soft blotting paper was placed and a light pressure (about 1 kilogram) was applied. After about 15 minutes the filter paper was carefully removed, and the plate was dried in front of a fan. Two perpendicular diameters of the zones of inhibited hemolysis were measured under magnification (9×) in a table projector. Different times and temperatures of incubation were tried. For the diffusion of samples into the staphylolysin containing gel 24 hours at +4° C was found to give optimal sharpness of the zones. The diffusion time of free staphylolysin into the erythrocyte containing gel was found to be a critical step. Too short (less than 20 minutes) or too long (more than 40 minutes) an incubation time was found to result in diffuse zones of inhibited hemolysis.

Staphylococcal antitoxin from Statens Seruminstitut, Copenhagen, Denmark, was used as reference. Double samples of a serial dilution containing 20, 10, 5, 2.5 and 1.25 U/ml

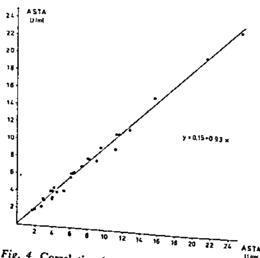


Fig. 4. Correlation between double determinations of 24 sera in the same plate. The correlation coefficient (r) was 0.99 and the regression line y = 0.15 + 0.93×.

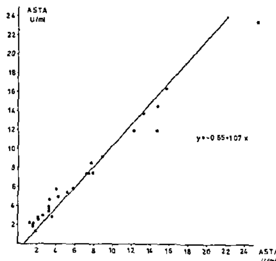


Fig. 5. Correlation between two determinations in two different plates. The correlation coefficient (r) was 0.99 and regression line y = −0.65 + 1.07×.

were used as a standard for each plate. The standard curve was constructed by plotting the sum of two perpendicular zone diameters against the ASTA-content (U/ml) in a semilogarithmic diagram (Fig. 2).

To assess the accuracy of the method 41 selected sera with elevated levels of ASTA were examined both with the gel-diffusion method and with the conventional tube-dilution method. Fig. 3 gives the correlation. The correlation coefficient (r) was found to be 0.95 and the regression line was y = 0.60 + 0.94×.

The *intraplate* standard deviation was determined with 25 estimations of ASTA on the same plate using two sera with an ASTA-content of 8.8 and 2.2 U/ml, respectively. The SD was calculated to be 0.55 and 0.15, respectively. Fig. 4 gives the correlation between double determinations of 24 sera in the same plate: the correlation coefficient (r) was 0.99 and the regression line y = 0.15 + 0.93×.

The *interplate* standard deviation was determined with the same sera as that used for determination of the intraplate standard deviation. The two sera were examined on different plates on 10 different occasions. Different batches of rabbit erythrocytes and staphylolysin containing gels were used each time. The SD was calculated to be 0.70 and 0.24, respectively. Fig. 5 shows the correlation between determinations of 24 sera on two different occasions. The correlation coefficient (r) was found to be 0.99 and the regression line was y = −0.65 + 1.07×.

DISCUSSION

A micro-method for routine determination of human antibodies to staphylococcal α-toxin (antistaphylolysin, ASTA) based on reversed single radial immunodiffusion is presented. The results obtained correlated well (r = 0.95) with the conventional tube-dilution method. The method described permits accurate determination of the ASTA content, an advantage compared with the tube-dilution method, in which the determinations are based on reading in double dilution steps. The low intra- and interplate standard deviations found for the gel-diffusion method presented further illustrate its usefulness. The gel-diffusion method is technically simple, it is time-saving (the time required for its performance is reduced by one third compared with the tube-dilution method) and the cost of the material for the gel-diffusion method is only about 50 per cent of that used for the conventional tube-dilution method.

As the test requires only small amounts of serum (10 μl), blood takes as capillary samples can be used. This makes the test suitable for determining ASTA in sera from newborn, infants and children.

This work was supported by grants from the *Medical Faculty, University of Lund,* and from *Alfred Österlunds Stiftelse.*

REFERENCES

1. *Brorson, J.-E., Holmberg, I. & Seeberg, S.:* A plate-diffusion method for the detection of antistaphylolysin activity of human serum. Acta path. microbiol. scand. Sect. B, *82:* 182–184, 1974.
2. *Neisser, M. & Wechsberg, F.:* Über das Staphylotoxin. Z. Hyg. Infekt.-Kr. *36:* 299–349, 1901.
3. *Zetterwall, O.:* Antibody activity in monoclonal immunoglobulin G. Acta Medica Scand., Suppl. 492, 1968.

Acta path. microbiol. scand. Sect. C, *83:* 459–468, 1975

PSEUDOMONAS AERUGINOSA INFECTION IN CYSTIC FIBROSIS

Humoral and Cellular Immune Responses Against Pseudomonas aeruginosa

NIELS HØIBY, VAGN ANDERSEN and GUNNAR BENDIXEN

Statens Seruminstitut, Department of Clinical Microbiology at Blegdamshospitalet, Department of Clinical Chemistry, Blegdamshospitalet; Paediatric Clinic TG with the Paediatric Clinic of Dronning Louises Børnehospital, and the Laboratory of Clinical Immunology, Medical Department TA, Rigshospitalet, Copenhagen, Denmark

Høiby, N., Andersen, V. & Bendixen, G. *Pseudomonas aeruginosa* infection in cystic fibrosis. Humoral and cellular immune responses against *Pseudomonas aeruginosa*. Acta path. microbiol. scand. Sect. C, *83:* 459–468, 1975.

Ten patients with cystic fibrosis (CF) chronically infected with mucoid *P. aeruginosa*, twelve CF patients without *P. aeruginosa* infection, and ten normal controls have been examined for humoral and cellular immune responses against *P. aeruginosa* by means of crossed immunoelectrophoresis, the lymphocyte blast transformation test, the leucocyte migration agarose test and an intracutaneous test. In addition to *P. aeruginosa* antigens, the cellular immune response to plant mitogens, *E. coli*, *C. albicans* and tuberculin was examined. Compared with the other two groups, CF patients chronically infected with mucoid *P. aeruginosa* presented significantly changed laboratory parameters indicating inflammatory reactions and humoral hyperimmunization. The results show that neither of the two groups of CF patients suffered from severe generalized immunodeficiency. However, in contrast to the pronounced *humoral* immune response against *P. aeruginosa* in chronically infected CF patients, the *cellular* immune response against *P. aeruginosa* was not significantly changed as compared with the two other groups of patients. The intracutaneous test with *P. aeruginosa* showed a marked difference between the groups of patients. In all chronically infected CF patients, a typical weal and flare reaction occurred, but such a reaction was not observed in the other groups of patients. No humoral or cellular immune response against mucoid substance from *P. aeruginosa* was found.

Key words: Cystic fibrosis, *Pseudomonas aeruginosa;* immune response; IgE; mucoid substance.

N. Høiby, Department of Clinical Microbiology, Hvidovre Hospital afs. 445, Kettegårds Alle 30, DK-2650 Hvidovre, Copenhagen, Denmark.

Received 9.xi.75 Accepted 2.vii.75

Chronic infection with mucoid strains of *P. aeruginosa* in patients (pts.) with cystic fibrosis (CF) (8, 13) is associated with a pronounced and heterogenous humoral immune response against these bacteria and with an increased number of circulating B and T cells (12, 14, 15, 18) A positive correlation between the concentration of circulating T cells and the number of different *P. aeruginosa* precipitins has been found in these pts., but little is known about the quality or functional capacity of T cells in CF (15).

The present study reports investigations of the cellular and the humoral immune re-

sponses against P. aeruginosa in CF pts. with chronic *P. aeruginosa* infection, CF pts. without *P. aeruginosa* infection and in control persons. Studies of the cellular immune response against other microbial antigens and the activation of lymphocytes by plant mitogens are included.

MATERIALS AND METHODS

Patients and Controls

(i) Ten CF pts. (5 males, 5 females, mean age: 12 years, range: 8–23 years) with chronic infection with mucoid *P. aeruginosa* (CF + P) and multiple precipitins in serum against these bacteria were examined. The pts. have been followed as previously described (12, 13). Mean duration of the *P. aeruginosa* respiratory tract infection: 3 years (range: 1–5 years). One of these pts. had also *S. aureus* in the respiratory tract at the time of the study. Routine studies of the lung function showed a reduced vital capacity and peak expiratory flow rate when compared with tables of normal values (23) (in average the values of CF + P pts. were 1.8 standard deviations (SD) below mean values applying to healthy normal persons of the same height.)

(ii) Twelve CF pts. (6 males, 6 females, mean age: 12 years, range: 9–19 years) without *P. aeruginosa* infection (CF — P) and without multiple *P. aeruginosa* precipitins in serum were also examined. In average the vital capacity of the lungs was 0.1 SD below mean normal values, and the *peak expiratory flow rate was 0.4 SD below mean* normal values (23). There were no significant differences between the CF + P and CF — P groups as regards mean weight and mean height, and in these respects the 2 groups were less than 1 SD below normal mean values applying to healthy persons of the same age Eight of the CF — P pts. had *S. aureus, H influenzae* or *D. pneumoniae* in the respiratory tract at the time *of the study.*

(iii) Ten control persons were examined (6 *males, 4 females, mean age 12 years, range· 8–19* years). They had no infectious or immunological disorders, 2 were not hospitalized and 8 were hospitalized for enuresis, constipation or headache.

Blood samples for the various examinations carried out in this study were in each case obtained simultaneously and the results were read independently and 'blindly Sera were stored at –30° C with NaN_3 added (15 mM

P. aeruginosa *Antigens*

1) Water-soluble antigens were obtained by sonication from 4 different O groups of *P. aeruginosa* (St-Ag) *as previously described* (12, 17). The present batch of St-Ag was passed through a 0.45 μm Millipore filter. The colloid concentration measured by refractometry (12) was 13.4 g/l. 2) Heat-killed whole *P. aeruginosa* cells representing the 4 O groups of St-Ag (equal amounts—wet weight—of the 4 strains). The bacteria were cul*tured and washed as described previously* (19); they were heat-killed at 70° C for 30 min and adjusted to 10^9 bacterial cells per ml. 3) Mucoid substance (dry weight = 1 mg/ml) obtained from a mucoid strain (O group 11) has been described previously (19); in addition, mucoid substance was prepared from 2 mucoid strains of *P. aeruginosa* isolated from 2 of the CF + P pts. of the present study. In some of the experiments, mucoid substance was further purified according to the ethanol precipitation method described by *Doggett et al.* (1964).

Lymphocyte Blast Transformation Studies

The lymphocyte transformation tests were carried out as detailed previously (1) the mitogens employed were phytohaemagglutinin (PHA), concanavalin A (Con A), and pokeweed mitogen (PWM) and the microbial antigens were whole, heat-killed *E. coli, Candida albicans* extract and tuberculin (PPD, Statens Seruminstitut) which have likewise been *described (1).* Heat-killed *P. aeruginosa* were used at a concentration of 10^8 per 0.5 ml culture with 10^6 lymphocytes; a series of tenfold dilutions of bacteria was used in each test. St-Ag was employed at a concentration of 2.7 μg/0.5 ml culture and tenfold dilutions thereof. Mucoid substance was added at a concentration of *50 μg/0.5 ml culture and tenfold dilutions thereof.* The highest concentration was determined for each preparation according to the results of experiments in which toxicity was revealed by the addition of PHA; the highest *concentration* used was that which gave a moderate depression of the response to PHA.

The blast transformation response was quantitated by the incorporation of ^{14}C-thymidine which was added *24 h before termination* (1). In unstimulated cultures, the average incorporation *per culture was 198 counts per min* (cpm) and was not significantly different in CF pts and controls. The unstimulated values have been subtracted from the values given for stimulated cultures.

Leucocyte Migration Studies

Cellular immunity as expressed by antigen-induced leucocyte migration inhibitory activity (2, 4, 5) was measured by the leucocyte migration agarose test (LMAT), as described by *Clausen* (1971) The method exposes *peripheral* blood leucocytes (granulocytes, lymphocytes, monocytes)

to antigen and measures antigen-induced alterations of their migratory capacity under an agarose layer.

Dextran sedimentated peripheral blood leucocytes and the agarose medium (Litex batch 055, Glostrup, Denmark) was prepared according to *Clausen* (1975).

Incubation with antigen: 1) St-Ag was used at a final concentration of 0.05 and 0.005 μg protein per 100 μl incubation medium. 2) Whole, heat-killed *P. aeruginosa* cells were used at a final concentration of 10^4, and 10^5 and 10^6 per 100 μl incubation medium. 3) Tuberculin (PPD, Statens Seruminstitut, Copenhagen) was used at a final concentration of 10 μg and 5 μg per 100 μl incubation medium. These antigen concentrations were selected because they were the highest concentrations without non-specific, toxic inhibitory activity, based on titrations in initial pilot experiments. Mucoid substance from *P. aeruginosa* was also tested in initial migration assays but was not included in the final experiments due to non-specific toxicity even at very low concentrations. The reason for this toxicity has not yet been found.

The antigen-containing solutions or comparable antigen-free control solutions were added to samples of leucocyte suspension according to schedule. The final cell concentration in the antigen incubation period was 2.1 × 10^8/ml. The cell suspensions were incubated at 37° C for 90 min. There was not enough blood to carry out all the LMAT experiments on all pts and controls.

After incubation of the leucocyte suspensions with antigen, 7 μl aliquots each containing 1.5 × 10^6 leucocytes were placed in the agarose gel holes. The agarose plates were placed at 37° C on 2 per cent (v/v) CO_2 in air saturated with water vapour, the pH of the gel being maintained at 7.2–7.4. After 24 h the migration areas were projected and measured planimetrically. The plate-to-plate and hole-to-hole variation coefficient between the migration areas of identically treated cultures was below 7 per cent. The migration index was calculated on the basis of quadruplicates as the ratio between the average of migration areas of antigen-containing cultures and average of migration areas of cultures without antigen.

Crossed Immunoelectrophoresis

Serum precipitins against *P. aeruginosa* (St-Ag) were analysed by means of crossed immunoelectrophoresis using microtechnique as described previously (14).

Detection of Antibodies in Serum against Mucoid Substance

The occurrence of antibodies against mucoid substance was examined by i) crossed immunoelectrophoresis of 2 μl mucoid substance against pts.'s sera according to previously described methods (14, 19). ii) Immunodiffusion studies in agarose: the diffusion was carried out as radial diffusion of 20 μl of pts. sera into agarose gel containing mucoid substance (20 μl/cm²). The technical details have been described previously (20). iii) Indirect haemagglutination according to *Doggett & Harrison* (1972). Human O Rh positive red blood cells were sensitized with mucoid substance as described by *Doggett & Harrison* (1972). Two drops of inactivated (56° C for 30 min) serum in 2-fold dilutions in phosphate buffered saline (pH 7.38) were mixed with 2 drops of 2.5 per cent sensitized red blood cells in phosphate buffered saline and the samples were incubated and read as described previously (11). iv) Indirect immunofluorescence microscopy: smears of mucoid substance on glass slides were fixed at 65° C for 2 h. The smears were then incubated with serum from the pts. for 30 min at room temperature in moist chambers, washed 2 times in phosphate buffered saline (pH 7.2) and incubated with fluoresceinisothiocyanate (FITC)-conjugated rabbit IgG, specific for human IgG, IgM, IgA and complement C3 (β_{1C}globulin) (Dakopatts, Copenhagen) as described previously, appropriate controls being included (15, 21). The specificities and working titres of the conjugates as well as the equipment of the microscope used have been described previously (15, 21).

Intracutaneous Reaction

After appropriate test in guinea pigs, the intracutaneous reaction against whole, heat-killed *P. aeruginosa* was studied in 10 normal persons, 9 CF + P, and 7 CF — P pts. Heat-killed *P. aeruginosa* (5 × 10^6 and in some additional tests 2.5 × 10^7 bacterial cells) suspended in 0.1 ml 0.154 M NaCl, were injected intracutaneously on the volar surface of the forearm, and the skin reaction was examined and measured after 20 min, 2 h, 6–8 h, 24 h, 48 h and 72 h. In all pts. and in most of the control persons, saline was administered simultaneously in the other arm as control. The skin tests were carried out after termination of the rest of this study; therefore, 5 of the CF + P pts., 6 of the CF — P pts. and all the control persons are persons other than those *in vitro* tested as described above.

Immunoglobulins, Acute Phase Proteins, Erythrocyte Sedimentation Rate and Leucocyte Concentrations

The concentrations in serum of IgG, IgA, IgM, haptoglobin and orosomucoid were determined by means of routine rocket-immunoelectrophoresis (22) using monospecific rabbit antibodies against human IgG etc. (Dakopatts, Copenhagen) and Standard-Human-Serum from Behringwerke (West

TABLE 1. *Particle Concentration of Leucocytes, Erythrocyte Sedimentation Rate (ESR) and the Concentrations of some Acute Phase Proteins in Peripheral Blood from Patients with Cystic Fibrosis with (CF + P) and without (CF — P)* Pseudomonas aeruginosa *Infection and from Control Persons (C)*

	Leucocytes ($\times 10^9$/l)	*Neutrophils* ($\times 10^9$/l)	*Lymphocytes* ($\times 10^9$/l)	ESR (mm)	*Haptoglobin* (g/l)	*Orosomucoid* (g/l)
CF + P mean	8.8	5.0	3.4	30	2.6	1.3
(A) range	(6.4–13.3)	(2.0–11.0)	(2.0–5 0)	(9–74)	(1.5–5.2)	(0.8–1 9)
CF—P mean	7.2	4.0	2.8	11	1.9	1.0
(B) range	(5.3–8.9)	(1.9–5.7)	(1.9–3.7)	(3–14)	(0.6–3.6)	(0.6–1.3)
C mean	5.9	3.1	2.5	10	1.5	1.0
(C) range	(4.9–7.2)	(2.2–3.8)	(1.9–3.2)	(1–30)	(0.6–3.6)	(0.8–1.6)
Significance of difference	A-C: $p<0.01$ B-C: $p<0.05$	A-C: $p<0.01$	A-C: $p<0.01$	A-C: $p<0.05$	A-C: $p<0.01$	A-C: $p<0.05$

TABLE 2. *Concentration of Immunoglobulins and Number of Precipitating Antibodies against* Pseudomonas aeruginosa *in Peripheral Blood from Patients with Cystic Fibrosis with (CF + P) and without (CF — P)* Pseudomonas aerugoinosa *Infection and from Control Persons (C)*

	Pseudomonas precipitins (number)	IgA (g/l)	IgE (10^3 U/l)	IgG (g/l)	IgM (g/l)
CF + P mean	26	2.8	94	15.6	1.1
(A) range	(15–48)	(1.0–8.0)	(29–228)	(11.1–20.7)	(0.6–2.2)
CF—P mean	0	1.8	81	11.7	0.9
(B) range	(0–2)	(0.2–2.9)	(42–222)	(9.2–14.5)	(0.4–1.5)
C mean	0	1.3	114	9.4	0 6
(C) range	(0)	(0.7–1.8)	(29–271)	(6.6–14)	(0.3–1.0)
Significance of difference	%	A–C: $p<0.05$	n.s.	A–C: $p<0.01$ B–C: $p<0$ 02	A–C: $p<0.01$

% calculations omitted as precipitins were among the selection criteria of the groups.
n.s.: not significant.

TABLE 3. *Lymphocyte Transformation Induced by Non-specific Mitogens Incubated with Cells from Peripheral Blood from Patients with Cystic Fibrosis with (CF + P) and without (CF — P)* Pseudomonas aeruginosa *Infection and from Control Persons (C)*

	PHA (10^3 counts/min)	Con A (10^3 counts/min)	PWM (10^3 counts/min)
CF + P mean	16.0	2.4	3.1
(A) range	6.1–21.3)	(0.4–6.9)	(0.5–5.9)
CF — P mean	17.8	2.5	3 8
(B) range	(8.5–34 1)	(0.5–8.8)	(1.3–7.2)
C mean	17 0	3.3	3 6
(C) range	(10 8 26.4)	(1.6–5.8)	(2.0–6 0)
Significance of difference	n s	n s.	n.s.

many) as standard. The particle concentration leucocytes was determined routinely by a Coul-S automatic electronic counter and the distri-tion was determined by conventional microscopy blood smear The erythrocyte sedimentation es were determined by conventional methods. e concentration of IgE in serum (kindly per-ned by dr. *Bent Weeke*, Rigshospitalet) was ermined by the radioimmunosorbenttest (Phade-IgE Test, Pharmacia, Uppsala, Sweden). The ults are given as Units per litre. One Unit is proximately 2 4 ng.

tistical Methods

The Mann-Whitney test and Spearman's corre-ion coefficient (6). Level of Significance: $p \leq$ 5 (double-tailed tests).

RESULTS

bles 1 & 2 show that the CF + P group th many *P. aeruginosa* precipitins differs nificantly from the control group as re-rds most parameters reflecting inflamma-ry and infectious processes and the humoral mune response; the concentrations of IgE re within normal limits. The CF — P group ows an intermediate position as regards ost of these values: only two of the para-eters were significantly different from those the control group and none of the para-eters were significantly different from those the CF + P group. In the CF + P group, e number of *P. aeruginosa* precipitins in rum showed a positive correlation to the ood concentration of leucocytes ($R = 0.83$, $p<0.01$) and to the blood concentration of neutrophils ($R = 0.81$, $p<0.01$), but not to the blood concentration of lymphocytes.

Table 3 shows that although the responses of some of the pts. from the CF groups were rather low, there was no significant difference between the groups as regards blast transformation after stimulation with plant mitogens.

Table 4 shows that lymphocytes from the CF groups as well as from the control group were stimulated to blast transformation by various microbial antigens. However, a significantly lower response to stimulation with *E. coli* was noted in the CF + P group compared with the CF — P group.

Table 5 shows that whole *P. aeruginosa* cells as well as St-Ag could induce blast transformation in lymphocytes from both of the CF groups and from controls. However, the response was low in most of the CF + P pts. although no significant difference between the groups was found. The optimal concentration of heat-killed *P. aeruginosa* was similar in the pts. and the controls, varying between 10^7 and 10^5/0.5 ml lymphocyte culture. Virtually no stimulation was caused by mucoid substance in any of the groups. This result could not be ascribed to inhibitory effects of mucoid substance because PHA as well as PPD could stimulate CF + P lymphocytes to blast transformation in the presence of mucoid substance. Regarding the CF + P

ABLE 4. *Lymphocyte Transformation Induced by some Microbial Antigens Incubated with Cells from ripheral Blood from Patients with Cystic Fibrosis with (CF + P) and without (CF — P)* Pseudomonas aeruginosa *Infection and from Control Persons (C)*

		Escherichia coli (10^3 counts/min)	*Candida albicans* (10^3 counts/min)	Tuberculin (PPD) (10^3 counts/min)
CF + P (A)	mean	0 7	1.1	0.7
	range	(0–1.5)	(0.2–4.1)	(0–2.3)
CF − P (B)	mean	2.1	1.9	0.4
	range	(0 4–7.6)	(0.5–3.7)	(0.1–1.5)
C (C)	mean	1.6	3.3	1.1
	range	(0.6 4.2)	(1.4–6 0)	(0.2–2.1)
Significance of difference		A B· $p<0.02$	n.s.	n.s.

TABLE 5. *Lymphocyte Transformation Induced by* Pseudomonas aeruginosa *Antigens Incubated with Cells from Peripheral Blood from Patients with Cystic Fibrosis with (CF + P) and without (CF—P)* Pseudomonas aeruginosa *Infection and from Control Persons (C)*

		Whole, heat-killed Pseudomonas cells (10^3 counts/min)	Water-soluble antigens (St-Ag) (10^3 counts/min)	Mucoid substance (10^3 counts/min)
CF + P	mean	1.3	0.3	0 04
(A)	range	(0.4–3.3)	(0–1.3)	(0–0.2)
CF — P	mean	1.9	0.8	0 04
(B)	range	(0.2–4.2)	(0–2.7)	(0–0.5)
C	mean	2.3	0.7	0.04
(C)	range	(1.6–3.5)	(0–2.3)	(0–0.4)
Significance of difference		n.s.	n s.	n s.

group, the transformation response obtained by heat-killed *P. aeruginosa* showed a positive correlation to the transformation response obtained by heat-killed *E. coli* (R = 0.94, $p<0.001$) and by *C. albicans* (R = 0.74, $p<0.01$), but not to the response obtained by PPD.

Extensive washing (8 times instead of 3 times) of the lymphocytes from one of the CF + P pts. with 48 *P. aeruginosa* precipitins (Fig. 1) in whom the transformation values were among the lowest of the group had virtually no influence on the transformation response after stimulation with the microbial antigens. Thus, the lymphocytes in this experiment did not present evidence of membrane-associated factors interfering with lymphocyte transformation. Likewise, no interfering factors in the serum of this CF + P pt. were demonstrated, as similar results were observed when lymphocytes from the pt. were incubated in the pt.'s own serum or in pooled standard serum. Moreover, when lymphocytes from a normal person were incubated with serum from this CF + P pt. instead of pooled standard serum no significant differences in the transformation results obtained by microbial antigens were observed.

The LMAT studies showed (Fig. 2) that leucocytes from only one CF + P pt., 2 CF — P pts., and 2 controls showed low migration indices when *P. aeruginosa* antigens were used, and there were no significant differences between the groups. In accordance with results of previous routine skin tests, controls as well as CF — P pts. and most of the CF + P pts. showed low migration indices when PPD was used as antigen.

One pt. from the CF + P group was examined six weeks after successful Tobramycin treatment of the *P. aeruginosa* infection. She was re-tested 50 days later when *P. aerugi-*



Fig 1. Crossed immunoelectrophoresis of 2 µl St-Ag against serum (15 µl per cm² in the second dimension gel) from a patient with cystic fibrosis and chronic *P. aeruginosa* infection Forty-eight precipitates are visible in this plate (First dimension electrophoresis: anode to the right Second dimension electrophoresis: anode at the top Staining, Coomassie brilliant blue).

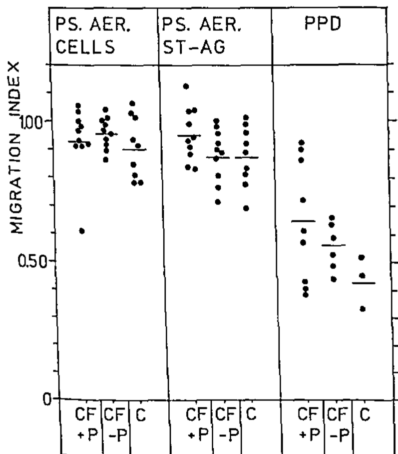


Fig. 2. Leucocyte migration agarose test with cells from peripheral blood from patients with cystic fibrosis with (CF + P) and without (CF — P) *P. aeruginosa* infection and from control persons (C). Influence of whole heat-killed *P. aeruginosa* cells (PS. AER. CELLS), water-soluble *P. aeruginosa* antigens (PS. AER. ST-AG) and tuberculin (PPD) The bars represent mean values.

nosa infection had become re-established. Neither the LMAT results nor the transformation test were significantly different from the rest of the CF + P group at any of these examinations.

In the group of normal controls, the intracutaneous test with heat-killed *P. aeruginosa* showed a hyperaemia of the skin, appearing within the first hour and increasing to 10–20 mm in diameter during the intitial 12–24 h. The hyperaemia disappeared after 24-48 h; a persisting central induration, if present, was always less than 5 mm. The CF — P group showed similar reactions The CF + P group, however, reacted in a completely different way. All these pts. showed an immediate type weal and flare reaction which appeared within the first 10 min after injection (Fig. 3). Controls with saline were always negative. Two hours after the injection, the reaction had always disappeared and a hyperaemia which was seen in the 2 other groups did not appear in the CF + P group. No signs of type III reaction or type IV reaction were observed. When the number of injected bacterial cells was increased, the described type of reactions was more pronounced, but no signs of type III or type IV reactions were noted.

Antibodies against mucoid substance were



Fig. 3. Skin-reaction 20 min after intracutaneous injection of 5×10^9 heat-killed *P. aeruginosa* cells in 0.1 ml physiological saline. A typical weal and flare reaction is seen. Control with saline was negative. The patient was a 14 years old boy with cystic fibrosis and chronic *P. aeruginosa* infection in the respiratory tract. His serum contained 48 precipitins against *P. aeruginosa* (Fig. 1) but serum IgE concentration was within normal limits (178 kU/l).

not revealed by any of the methods used, either in sera from the CF + P group or in sera from the two other groups.

DISCUSSION

The present study confirms that laboratory parameters are significantly changed in CF pts. with persistent infection with mucoid *P. aeruginosa* indicating inflammatory reactions and humoral hyperimmunization (16). CF pts. without chronic *P. aeruginosa* infection present less abnormal values.

As to the cellular immune response, the results of the lymphocyte transformation studies using plant mitogens and the LMAT studies using PPD show that neither CF + P nor CF — P suffer from severe generalized cellular immunodeficiency. This is in accordance with recently published results obtained by PHA and PWM in transformation tests (24).

However, in spite of the chronic *P. aeruginosa* infection, neither the lymphocyte transformation studies, nor the LMAT or intracutaneous tests revealed any significant increase in the cellular immune response against *P. aeruginosa* in CF + P pts. compared to the 2 other groups. On the contrary, in the transformation studies, the CF + P pts. tended to give lower responses with *P. aeruginosa* antigens and with antigens from *E. coli* and *C. albicans* as compared with the other two groups. Moreover, in the CF + P group an interesting correlation between the response to stimulation with whole heat-killed *P. aeruginosa* cells and the response with *E. coli* and *C. albicans* was observed. Further studies are needed to elucidate whether these correlations are related to cross-reacting antigens (17, 18) or merely reflect a generalized, slight depression of the activity of lymphocytes or phagocytic cells necessary for the transformation reaction. Serum factors interfering with the transformation response are also a possibility, but this seems less likely considering the results obtained in the present study.

It was not possible to reveal humoral or cellular immune responses against mucoid substance in any of the pts. This can of course be due to technical reasons as antibodies against mucoid substance have been reported from another laboratory (8), but other explanations are poor immunogenicity of mucoid substance or a state of tolerance against mucoid substance in our CF + P pts. If so, the selective advantage as infectious agent of mucoid strains compared with nonmucoid *P. aeruginosa* strains in CF + P is obvious as previously discussed (12, 14). Experiments to elucidate this question are in progress.

Although not reflected in the total serum IgE concentrations, the immediate type skin reaction to *P. aeruginosa* in CF + P pts. most probably indicate a specific reaction between *P. aeruginosa* and homocytotropic antibodies followed by liberation of vasoactive amines. However, the possibility of complement-mediated release of histamine from leucocytes cannot be ruled out (10). Whether such reactions in the lungs of CF + P pts. may contribute to the pathophysiology of the infection remains to be investigated, but the possibility is suggested by recently published results (3, 9).

This work was supported by the *Thorvald Madsen Legat*, the *Danish Medical Research Council*, the *National Danish Association against Cystic Fibrosis*, the *Danish Hospital Research Foundation, Region of Copenhagen, Greenland and the Faroe Islands*, and the *P. Carl Petersens Foundation*. We thank *Anni Bethien, Vita Weibull* and *Aase Bavngaard* for skilful technical assistance.

REFERENCES

1. *Andersen, V., Thomsen, M. & Cohn, J.:* Immune deficiency in sex-linked hereditary thrombocytopenia. Acta path. microbiol. scand. Sect. C, *83:* 177–183, 1975.
2. *Bendixen, G. & Søborg, M.:* A leucocyte migration technique for in vitro detection of cellular (delayed type) hypersensitivity in man. Dan. Med. Bull. *16:* 1–6, 1969
3. *Brogan, T. D., Ryley, H. C., Neale, L. & Yassa, J.:* Soluble proteins of bronchopulmonary secretions from patients with cystic fibrosis, asthma and bronchitis. Thorax *30:* 72–79, 1975.
4. *Clausen, J. E.:* Tuberculin-induced migration inhibition of human peripheral leucocytes in agarose medium. Acta allergol. *26:* 56–80, 1971.
5. *Clausen, J. E.:* The agarose migration inhibition technique for in vitro demonstration of cell-mediated immunity in man. A review. Thesis, University of Copenhagen 1975, p. 1–45.
6. *Diem, K.* (ed.): *Documenta Geigy Scientific Tables*. J. R. Geigy S. A. Basle 1962, pp. 66–67 & 124–127.
7. *Doggett, R. G., Harrison, G. M. & Wallis, E. S.:* Comparison of some properties of *Pseudomonas aeruginosa* isolated from infections in persons with and without cystic fibrosis. J. Bacteriol. *87:* 427–431, 1964.
8. *Doggett, R. G. & Harrison, G. M.: Pseudomonas aeruginosa.* Immune status in patients with cystic fibrosis. Infect. and Immun. *6.* 628–635, 1972.
9. *Gordon, D. S., Hunter, R. G., O'Reilly, R. J. & Conway, B. P.: Pseudomonas aeruginosa* allergy and humoral antibody-mediated hypersensitivity pneumonia. Am. Rev. Resp. Dis. *108:* 127–131, 1973.
10. *Grant, J. A., Dupree, E., Goldman, A. S., Schultz, D. R. & Jackson, A. L.:* Complement-mediated release of histamin from human leucocytes. J. Immunol. *114:* 1101–1106, 1975.
11. *Hoff, G. E. & Høiby, N. Staphylococcus aureus* in cystic fibrosis. Antibiotic sensitivity and phage types during the latest decade. Investigations of the occurrence of protein A and some other properties of recently isolated strains in relation to the occurrence of precipitating antibodies. Acta path. microbiol. scand. Sect. B, *83:* 219–225, 1975.
12. *Høiby, N. & Axelsen, N. H.:* Identification and quantitation of precipitins against *Pseudomonas aeruginosa* in patients with cystic fibrosis by means of crossed immunoelectrophoresis with intermediate gel. Acta path. microbiol. scand. Sect. B, *81:* 298–308, 1973.
13. *Høiby, N.:* Epidemiological investigations of the respiratory tract bacteriology in patients with cystic fibrosis. Acta path. microbiol. scand. Sect. B, *82:* 541–550, 1974.
14. *Høiby, N.: Pseudomonas aeruginosa* infection in cystic fibrosis. Relationship between mucoid strains of *Pseudomonas aeruginosa* and the humoral immune response. Acta path. microbiol. scand. Sect. B, *82:* 551–558, 1974.
15. *Høiby, N. & Mathiesen, L.: Pseudomonas aeruginosa* infection in cystic fibrosis. Distribution of B and T lymphocytes in relation to the humoral immune response. Acta path. microbiol. scand. Sect. B, *82:* 559–566, 1974.
16. *Høiby, N., Jacobsen, L., Jørgensen, B. A., Lykkegaard, E. & Weeke, B.: Pseudomonas aeruginosa* infection in cystic fibrosis. Occurrence of precipitating antibodies against *Pseudomonas aeruginosa* in relation to the concentration of sixteen serum proteins and the clinical and radiographical status of the lungs. Acta Pædiatr. Scand. *63:* 843–848, 1974.
17. *Høiby, N.:* Cross-reactions between *Pseudomonas aeruginosa* and thirty-six other bacterial species. Scand. J. Immunol. *4:* Suppl. 2, 187–196, 1975.
18. *Høiby, N.:* Normally occurring precipitating antibodies against *Pseudomonas aeruginosa.* Prevalence specificities and titres. Scand. J. Immunol. *4:* Suppl. 2, 197–202, 1975.
19. *Høiby, N.:* The serology of *Pseudomonas aeruginosa* analysed by means of quantitative immunoelectrophoretic methods. I. Comparison of thirteen O groups of *Pseudomonas aeruginosa* with a polyvalent *Ps. aeruginosa* antigen-antibody reference system. Acta path. microbiol. scand. Sect. B, *83:* 321–327, 1975.
20. *Koch, C. & Høiby, N.:* Nitroblue-tetrazolium staining of human neutrophil granulocytes. Effects of stimulation with *Pseudomonas* antigens in the presence or absence of human *Pseudomonas* precipitins. Acta path. microbiol. scand. Sect. B, *81:* 787–794, 1973.
21. *Koch, C., Høiby, N. & Wiik, A.:* Nitroblue-tetrazolium (NBT)-reduction by human peripheral neutrophil granulocytes in the presence of bacterial antigens. Evidence for

immune complex ingestion. Acta path. microbiol. scand. Sect. C, *83:* 144–156, 1975.

22. *Laurell, C.-B.:* Electroimmuno assay. Scand. J. Clin. Lab. Invest. *29:* Suppl. 124, 21–37, 1972.
23. *Thuesen Pedersen, J., Lykkegaard, E., Færø, O. & Jacobsen, L.:* Lung function in children and young adults with cystic fibrosis. Dan. Med. Bull. *21:* 12–19, 1974.
24. *Wallwork, J. C., Brenchley, P., McCarthy, J., Allan, J. D., Moss, D., Ward, A. M., Holzel, A., Williams, R. F. & McFarlane, H.:* Some aspects of immunity in patients with cystic fibrosis. Clin. exp. Immunol. *18:* 303-320, 1974.

Acta path. microbiol. scand. Sect. C, *83:* 469–475, 1975

PRECIPITATING ANTIBODIES AGAINST *PSEUDOMONAS AERUGINOSA* IN SPUTUM FROM PATIENTS WITH CYSTIC FIBROSIS: SPECIFICITIES AND TITRES DETERMINED BY MEANS OF CROSSED IMMUNOELECTROPHORESIS WITH INTERMEDIATE GEL

P. O. SCHIØTZ and N. HØIBY

Statens Seruminstitut, Department of Clinical Microbiology at Hvidovre Hospital, and the Paediatric Clinic TG with the Paediatric Clinic of Dronning Louises Børnehospital, Rigshospitalet, Copenhagen, Denmark

Schiøtz, P. O. & Høiby, N. Precipitating antibodies against *Pseudomonas aeruginosa* in sputum from patients with cystic fibrosis: specificities and titres determined by means of crossed immunoelectrophoresis with intermediate gel. Acta path. microbiol. scand. Sect. C, *83:* 469–475, 1975.

By means of crossed immunoelectrophoresis with intermediate gel, paired 24-h samples of sputum and sera from 19 patients with cystic fibrosis have been examined for the occurrence of precipitins against *Pseudomonas aeruginosa*. Saliva from 9 of the patients was subject to similar analyses. A total of 50 precipitins was found in fifteen of the sputa and 296 precipitins were found in 18 of the sera. Fourteen of the patients had precipitins in sputum as well as in serum; one patient had a precipitin in sputum, but none in serum; and 4 had precipitins in serum, but not in sputum. All the precipitins in sputa could be identified and quantitated in relation to the reference system and, in this way, 16 different precipitins were found in the sputa. If present in sputum, titres of these precipitins were nearly always found to be lower in sputum than those of the corresponding precipitin in serum from one and the same patient. One of the precipitin was, however, found in 8 of the sputa, but not in the corresponding sera. There was a positive correlation between the number of precipitins per sputum and between titre of the strongest precipitins and number of precipitins per serum. Pseudomonas antigens were demonstrated in 3 of the sputa. Saliva from the patients contained rarely precipitins.

Key words: Cystic fibrosis; *Pseudomonas aeruginosa;* immunoelectrophoresis; sputum; immune response.

P. O. Schiøtz, Paediatric Department TG with the Paediatric Department of Dronning Louises Børnehospital, Rigshospitalet, Tagensvej, DK-2200 Copenhagen N. Denmark.

Patients (pts.) with cystic fibrosis (CF) and chronic *Pseudomonas aeruginosa* (*P. aeruginosa*) infection in the respiratory tract are characterized by a pronounced and heterogenous humoral immune response against these bacteria (4, 6). The antibodies circulating in patients' sera, however, do not promote immune elimination of the bacteria although the humoral immune response possibly keep the infection localized to the respiratory tract (6).

These findings invite investigations of the local immune response in the respiratory tract of CF pts. Previously, immunopathological studies (9) as well as studies of the concentrations of immunoglobulins and other antibacterial factors in sputum of CF pts. have not revealed any deficiencies in this respect (2). However, neither the concentration nor the specificities of antibacterial antibodies against *P. aeruginosa* in sputum from CF pts. have been investigated.

Crossed immunoelectrophoresis with intermediate gel (CIWIG) offer the possibility in one and the same operation to identify and quantitate precipitating antibodies even in complex antisera (4). Sputum can be used in quantitative immunoelectrophoretic methods if the sol phase is separated from the gel phase and the former used instead of serum (11, 12). Consequently, CIWIG was used in the present study to investigate the sol phase of sputum from CF pts. for the occurrence, identity and titre of precipitating antibodies against *P. aeruginosa*. Moreover, the same technique was used to investigate sera as well as saliva from the same pts. to see whether there is any correlation between the presence of antibodies against *P. aeruginosa* in these fluids and in serum

MATERIALS AND METHODS

Patients

Nineteen CF pts. were included in the study, 10 males and 9 females. These pts are followed in the CF Clinic of Rigshospitalet, Copenhagen, as described previously (4, 5). Mean age 13 years, range. 6 27 years. Fourteen of the pts were chronically (5) infected with mucoid strains of *P. aeruginosa*, one was intermittently (5) infected with a nonmucoid strain and one was intermittently (5) infected with a mucoid strain. These 16 pts. are denoted CF+P. Three of the pts. (CF–P) were not harbouring *P. aeruginosa*.

24-h-sample of Sputum

A 24-h-sample of sputum was collected from each pt. The pts. were instructed not to spit in the containers but to expectorate only after coughing. The containers were kept in a thermo-box at the pts.' bedside at 4° C throughout the 24 h.

Each sample was examined bacteriologically by microscopy and culture as previously described (5). The origin of the specimens from the lower respiratory tract was confirmed by the presence of respiratory epithelial cells and the absence of significant numbers of squamous epithelial cells, as described previously (5).

The separation of the sol phase of sputum was done by centrifugation at 3000 × *g* for 30 min at 4° C to remove air bubbles and saliva, the latter separating as a fluid layer above the gel (1). The saliva contamination of the sputum samples was macroscopically evaluated to range from 0–10 per cent. The samples were then centrifuged at 120000 × *g* (maximum value) at 4° C for 4 h. in the SW-40 Ti rotor, model I 2 – 65B Beckmann preparative ultracentrifuge The supernatant (sol phase) was stored in small aliquots at –80° C (10).

1-h-sample of Unstimulated Saliva

One hour unstimulated, mixed saliva samples (1–3 ml) were obtained from 9 of the patients included in the study. The saliva samples were centrifuged and stored as described for sputum (11).

Serum

Serum was obtained from each pt. on the day when sputum was collected and stored at –30° C with NaN_3 added (15 mM).

Crossed Immunoelectrophoresis with Intermediate Gel

The occurrence, specificities and titres of precipitating antibodies against *P. aeruginosa* were investigated by means of crossed immunoelectrophoresis with intermediate gel by microtechnique using a polyvalent *P. aeruginosa* Standard-Antigen (St-Ag)/Standard-Antibody (St-Ab) reference system, as previously described in detail (4, 7, 8). The concentration of serum, sputum or saliva in the intermediate gel was 40 µl/cm². The identity and titre of human precipitins in serum, sputum or saliva were determined in relation to the refe-

ABLE 1. *Occurrence of Pseudomonas Precipitins in Serum or Sputum of Patients with Cystic Fibrosis with (CF+P) or without* Pseudomonas aeruginosa *Infection (CF-P)*

Group of subjects	No. of pts.	No. and percentage of pts. with precipitins in serum	in sputum
CF (Total)	19	18 (95 %) (95 % c.l.: 74–100 %)	15 (79 %) (95 % c.l.: 54–94 %)
CF+P	16	16	13
CF-P	3	2	2

c.l.: confidence limits.

TABLE 2 *Mean ($\bar{x}$) and Range (r) of No. of Precipitins per Patient with Cystic Fibrosis and Precipitins in Serum and Sputum*

Group of subjects	Mean and range of No. of precipitins per pt. in serum	in sputum
CF (Total)	$\bar{x}$: 16.4 r: 1–36	$\bar{x}$: 3 3 r. 1–10
CF+P	$\bar{x}$: 18.4 r: 1–36	$\bar{x}$: 3.6 r: 1–10
CF-P	$\bar{x}$: 1 r: 1	$\bar{x}$: 1.5 r: 1–2

Abbreviations as in Table 1.

ence pattern, according to previously described methods (4, 7) and the quantitation was done by comparing the area including human precipitins with the area of corresponding rabbit precipitins (St-Ab) in 5 standard plates The standard plates were run exactly as described above, but instead of human fluids in the intermediate gel, the standard plates included rabbit antiserum (St-Ab) in the intermediate gel, as described previously (4, 7). The same titre classes as those previously described (7) were employed in the present study 0 µl/cm² < *titre 1* ≦ 1 µl/cm² < *titre 2* ≦ 5 µl/cm² < *titre 3* ≦ 10 µl/cm² < *titre 4* ≦ 20 µl/cm² < *titre 5* ≦ 40 µl/cm² < *titre 6* ≦ 80 µl/cm² < *titre 7.* A control in which the intermediate gel contained saline was always included every testday. In some instances, additional plates with lower concentration of sputum in the intermediate gel were run to facilitate the identification of the precipitins (Fig. 1 B and C).

Statistical Methods

Wilcoxon's test for pair differences and Spearman's correlation coefficient R (3) Level of significance 5 per cent (double-tailed tests)

RESULTS

It appears from Table 1 that 18 of the pts. had precipitins against *P. aeruginosa* in serum; 14 of these pts. had also precipitins in sputum whereas one pt. had one precipitin in sputum and no precipitins in serum. It is seen from Table 2 that, on an average, the number of precipitins in serum was much higher than the number of precipitins in sputum ($p < 0.01$). Examples are given in Fig. 1 A, B & C. Only two of the nine pts. examined (22 per cent) had precipitins in saliva (one and two precipitins, respectively) although some of these pts. had multiple precipitins in serum as well as in sputum.

All the *P. aeruginosa* precipitins in the sputum and saliva could be identified and quantitated in relation to the reference system. These precipitins represented 16 different specificities (Table 3). Precipitins numbers 2, 10, 15A and 38A were most frequent, accounting for 36 of the 50 precipitins in the sputa. In all pts. whose sputum contained precipitins, precipitins of the same specificities as those found in sputum could also be identified in serum and, in addition, precipitins of other specificities were also present in serum from 16 of the pts. One exception,

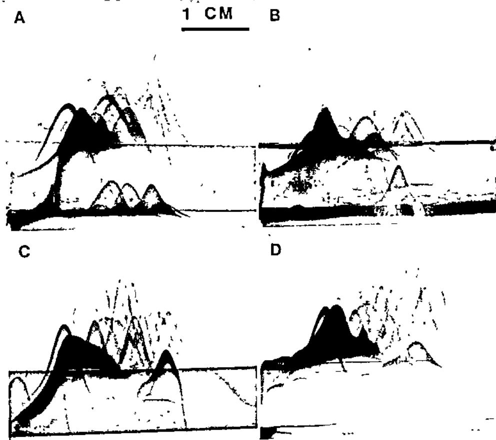


Fig. 1. Crossed immunoelectrophoresis with intermediate gel of 2 μl Pseudomonas antigen (St-Ag) in the well against rabbit antibodies against St-Ag (St-Ab) in the second dimension reference gel (20 μl per cm²). (Technical: 1 dim. electrophoresis: anode to the right, 2. dim electroph.: anode at the top. Staining: Coomassie brilliant blue).
A: The intermediate gel contains serum (40 μl per cm²) from a CF patient chronically infected with *P. aeruginosa.* Thirty-three precipitates could be revealed in this serum.
B: The intermediate gel contains sputum (40 μl per cm²) from the same patient as in Fig. 1 A. Ten precipitates could be revealed in this sputum.
C Same as Fig. 1 B, but the concentration of sputum in the intermediate gel is only 10 μl per cm² to facilitate the identification of the precipitates. The plates on Fig. 1 A, B & C were compared with a control plate (*1 D*) containing saline instead of serum or sputum in the intermediate gel; in this way, all 10 precipitates in sputum from the patient could be identified and precipitates of the same specificities could be identified in the patient's serum
D: Control plate with saline in the intermediate gel.

however, was noticed: Precipitin number 2 was found in 11 of the sputa but only in 3 of the corresponding sera, moreover, in 3 of these 11 sputa, no other precipitins could be revealed. The titres of the precipitins are given in Table 4. The geometrical mean of the titres in sera was 5.2 compared to 4.0 in sputa ($p<0.01$); among the 16 precipitin specificities, 15 were found in titres in serum higher than or equal to those in sputum. In contrast to this, the titre of precipitin number 2 in serum was either lower than or, in 3 cases, equal to the titre in sputum. The 3 precipitins found in saliva belonged to titre-class 1 and precipitins of the same specificities belonging to higher titre-classes were found in sputum and serum of these pts. A difference in number and titres of precipitins

TABLE 3. *Prevalences of the Different Pseudomonas Precipitins in Serum, Sputum and Saliva of Patients with Cystic Fibrosis*

Precipitin specificities*	No. of pts. with precipitins in serum (19 pts.)	in sputum (19 pts.)	in saliva (9 pts.)
2	3	11	
6	1	1	
7	1	1	
9	1	1	
10	9	9	2
14	2	2	
15A	7	7	
18	1	1	
38A	9	9	1
39	1	1	
40	2	2	
41	1	1	
44	1	1	
46	1	1	
48	1	1	
50	1	1	

* Enumeration of precipitins according to the previously published St-Ag/St-Ab reference system (8).

in pts. belonging to the CF+P group and in those in the CF-P group was noticed (Table 2) but these differences cannot be confirmed until larger groups of pts. have been studied.

Correlation between the number of precipitins in serum and sputum of the pts. was found to be positive (Fig. 2). However, the correlation coefficient is rather low because some pts. presented a relatively high number of precipitins in serum as compared to that in sputum.

If all the precipitin specificities of sputum were considered, any correlation between the number of precipitin and the titre would

TABLE 4 *Distribution of Pseudomonas Precipitins of the Titre-Classes*

Titre-class	No. of precipitins in serum	in sputum
7	7	9
6	12	3
5	13	9
4	6	9
3	2	4
2	2	9
1	0	7

Precipitins in serum are only included in this table if precipitins of the same specificities are found in sputum from the same patient.

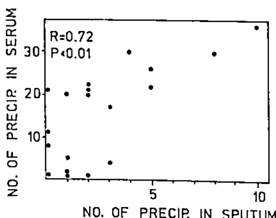


Fig. 2. Relationship between the number (NO.) of Pseudomonas precipitins in serum and in sputum of patients with cystic fibrosis. Spearman's correlation coefficient R and the corresponding p-value is given in the figure.

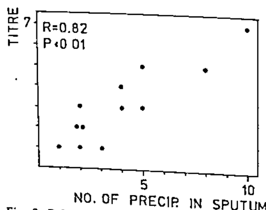


Fig. 3. Relationship between the number (NO.) of Pseudomonas precipitins and the titre of the strongest precipitin per individual sample of sputum from patients with cystic fibrosis in whom sputum contained precipitins. Spearman's correlation coefficient R and the corresponding p-value is given in the figure. Only precipitins corresponding to 15 out of the 16 specificities are included in this figure (see text).

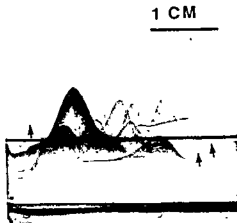


Fig. 4. Crossed immunoelectrophoresis with intermediate gel of 2 µl St-Ag in the well against St-Ab in the second dimension reference gel (20 µl per cm²). The intermediate gel contains sputum (40 µl per cm²) from a patient with cystic fibrosis. No antibodies could be revealed in this sputum, but 3 straight lines representing Pseudomonas antigens in sputum of the pt. can be seen parallel to the border of the intermediate gel (arrows). Due to electro-endosmosis, 2 of these lines are seen in the anodic part of the intermediate gel whereas the 3rd line is seen in the cathodic part of the reference gel. Reaction of identity between 2 of these precipitate lines and 2 of the precipitates of the reference pattern could be seen. Technique as in Fig. 1.

not be observed. Precipitin number 2 was different from the 15 other precipitin specificities as regards correlation to precipitins in serum, both with respect to prevalence and titres as outlined above. Thus, if precipitin number 2 were left out of consideration, the titres of the rest of the precipitins and the number of precipitins in sputum would be positively correlated (Fig 3). In 3 of the sputa, "free" antigens were demonstrated (Fig. 4). In one of these sputa, 3 antigens were found whereas only one antigen could be revealed in each of the remaining 2 sputa. None of these 3 pts. had demonstrable precipitins corresponding to these antigens in serum or sputum, although precipitins corresponding to other antigen specificities in the St-Ag/St-Ab reference system were present in these 3 sera and in two of the 3 sputa.

DISCUSSION

The present work shows that the methods used by *Ryley* & *Brogan* (2, 11, 12) who applied the sol phase of sputum to quantitative immunoelectrophoretic methods, are well-suited for a study of the occurrence of precipitins against *P. aeruginosa.*

In studies of sputum, however some sources of errors must be considered. First, the activity of proteolytic enzymes must be avoided. This was done by keeping the sputum at 4° C or lower from the moment of expectoration and throughout the period of preparation. The origin of sputum from the lower respiratory tract and the absence of blood in sputum were secured by microscopy as described previously (5).

The presence of a minor amount of saliva in sputum is probably unavoidable. In pts. with CF, saliva represented only a minor problem (below 10 per cent of the total volume as judged by inspection after the initial centrifugation). This is in accordance with the experience of *Brogan et al.* (1975). Moreover, the error induced by saliva could only be a minor dilution of the sputum (which is not very likely considering the viscous nature of CF sputum) leading to a slight fall in titre and number of the precipitins, as the results indicated that precipitins against *P. aeruginosa* are not excreted to any important degree by the salivary glands. The results of the present study show that CF pts with *P. aeruginosa* infection and many precipitins in serum against this bacteria frequently also have precipitins in sputum. In most cases these precipitins are fewer and of lower titres than those in simultaneously obtained sera. However, precipitin number 2 was frequently found in sputum and in all cases in higher titre in sputum than in serum. Whether this can be due to local production in the respiratory tract of this precipitin remains to be settled, but the present evidences suggest this possibility. Precipitin number 2 was also found in sputum from 2 of the CF-I pts. and in one of these, precipitin number 10 was also present in sputum. The latter

precipitin corresponds to a cross-reactive *P. aeruginosa* antigen (7), whereas antigen number 2 has not been shown to be cross-reactive. The significance of these results must await further investigation.

The origin and immunoglobulin classes of the other precipitins in sputum against *P. aeruginosa* cannot be determined on the basis of the results obtained in the present study. It is known that IgG as well as IgA is present in sputum from CF pts. and a local production of these antibodies in the respiratory tract of CF pts. has been demonstrated (9).

In the presence of infection, however, a filtration of serum proteins through the inflamed mucous membrane of the respiratory tract to sputum in CF pts. is certainly also a possibility to be taken into consideration since it may contribute substantially to the final amount of immunoglobulins in sputum (2).

To elucidate these problems it seems necessary to correlate the presence of *P. aeruginosa* precipitins in serum and sputum with the concentration of other proteins, including albumin, and the total concentration of immunoglobulins, especially as the number of detectable Pseudomonas precipitins decrease when dilutions of serum are studied. Such studies might also explain the differences between pts., especially why some of the pts. in the present study in whom many precipitins were demonstrable in serum presented only few, low-titred precipitins in sputum.

This work has been supported by grants from *Thorvald Madsens Legat, Landsforeningen til Bekæmpelse af Cystisk Fibrose* and *Statens Lægevidenskabelige Forskningsråd*. Mrs. *Annie Bethien* is thanked for skilful technical assistance.

REFERENCES

1. *Brogan, T. D., Ryley, H. C., Allen, Lynne & Hutt, Hilary:* Relation between sputum sol phase composition and diagnosis in chronic chest diseases. Thorax *26:* 418–423, 1971.
2. *Brogan, T. D., Ryley, H. C., Neale, Lynne & Yassa, Janet:* Soluble proteins of bronchopulmonary secretions from patients with cystic fibrosis, asthma and bronchitis. Thorax *30:* 72–79, 1975.
3. *Diem, K.* (Ed.): Documenta Geigy Scientific Tables. J. R. Geigy S. A., Basel 1962, pp. 66–67 & 124–127.
4. *Høiby, N. & Axelsen, N. H.:* Identification and quantitation of precipitins against *Pseudomonas aeruginosa* in patients with cystic fibrosis by means of crossed immunoelectrophoresis with intermediate gel. Acta path. microbiol. scand. Sect. B, *81:* 298–308, 1973.
5. *Høiby, N.:* Epidemiological investigations of the respiratory tract bacteriology in patients with cystic fibrosis. Acta path. microbiol. scand. Sect. B, *82:* 541–550, 1974.
6. *Høiby, N.: Pseudomonas aeruginosa* infection in cystic fibrosis. Relationship between mucoid strains of *Pseudomonas aeruginosa* and the humoral immune response. Acta path. microbiol. scand. Sect. B, *82:* 551–558, 1974.
7. *Høiby, N.:* Normally occurring precipitating antibodies against *Pseudomonas aeruginosa*. Prevalence specificities and titres. Scand. J. Immunol *4*, Suppl. 2, 197–202, 1975.
8. *Høiby, N.*. The serology of *Pseudomonas aeruginosa* analysed by means of quantitative immunoelectrophoretic methods. III. Reproducibility of a polyvalent *P. aeruginosa* reference standard-antigen. Acta path microbiol. scand. Sect. B, *83:* 433–442, 1975.
9. *Martinez-Tello, F. J., Braun, D. C. & Blanc, W. A.* Immunoglobulin production in bronchial mucosa and bronchial lymph nodes, particularly in cystic fibrosis of the pancreas. J. Immunol. *101:* 989–1003, 1968.
10. *Ryley, H. C. & Brogan, T. D.:* Variation in the composition of sputum in chronic chest diseases. Brit. J. exp. path. *49:* 625–633, 1968.
11. *Ryley, H. C.:* An immunoelectrophoretic study of the soluble secretory proteins of sputum. Biochim. Biophys. Acta. *271:* 300–309, 1972.
12. *Ryley, H. C. & Brogan, T. D.:* Quantitative immunoelectrophoretic analysis of the plasma proteins in the sol phase of sputum from patients with chronic bronchitis. J. Clin. Path. *26:* 852–856, 1973.

Received 16.vi.75 Accepted 15.vii.75

ADVICE TO AUTHORS

Authors must note and adopt the ACTA's customary arrangement and style; failure to do so may lead to delay in publication.

Instructions to authors are available on request to the Editorial Board. Manuscripts should be submitted to the national editor in their final form as top pages, not carbon copies, in double-spaced type-script in English, French, or German. All written matter, illustrations, and references should be submitted at the same time.

Ordinary articles should generally not exceed 5 printed pages and not more than 5 pages of illustrative material. They must contain a summary in English not exceeding 250 words. *Brief reports* for immediate publication must not exceed 1½–2 printed pages. Such reports will be published as soon as possible after receipt. Manuscripts will be reviewed by appropriate experts. Since manuscripts will not be insured against loss or damage, contributors are expected to retain duplicate copies of all material submitted for publication. Only illustrations of reasonable technical standards will be accepted. If the limit of 5 pages is exceeded and, if corrections in the proof are particularly numerous or the tabular and illustrative material unusually excessive and/or expensive, authors will be requested to contribute to the cost of publication. Reference to literature should conform to the standards of World Medical Periodicals or *World List of Scientific Periodicals*. In general, reviews of a topic unsupported by original observations will not be accepted.

The official abbreviation: Acta path. microbiol. scand. Sect. *A*, *B* or *C*.

Supplements: The publication of supplements will be governed by special rules which can be obtained from the Editor.

Articles accepted for publication in Section C

A. Jespersen: Potency of purified tuberculin determined by the shock method on hamsters.

O. Marker, G. Thörner Andersen & M. Volkert: The interplay between target organ concentrations of lymphocytic choriomeningitis virus and cell mediated immunity in baby mice.

S.-E. Svehag & D. Burger: Isolation of C1q-binding immune complexes by affinity chromatography and desorption with a diaminoalkyl compound.

B. Haneberg & C. Endresen: Fragments of immunoglobulins in human feces.

A. Ødegaard & J. Lamvik: The effect of phenulbutazone and chloramphenicol on phagocytosis of radiolabelled *Candida albicans* by human monocytes cultured *in vitro*.

J. N. Wiig: Surface structures influencing the migration *in vivo* (ecotaxis) of murine thymocytes, T- and B-cells.

B. G. Hansson: Evaluation of three reverse passive haemagglutination methods and two radioimmunoassay tests to be used for the detection of hepatitis B surface antigen.

R. Winsnes, T. Midtvedt & A. Trippestad: Rat intestinal glycoprotein lowering bactericidal activity of serum on ^{32}P-labelled *E. coli*.

J. N. Bruun, C. O. Solberg, E. Hamre, C. J. Janssen, S. Tunold & J. Eide: Acute disseminated phycomycosis in a patient with impaired neutrophil granulocyte function.

T. Midtvedt & A. Baardsen: Elimination of ingested ^{32}P-labelled *E. coli* from rat polymorphonuclear neutrophils (PMN). Evaluation of a method.

C. O. Solberg, K. E. Christie, B. Larsen & O. Tønder: Influence of antibodies and thermolabile serum factors on the bactericidal activity of human neutrophil granulocytes.

K. E. Christie, C. O. Solberg, B. Larsen, A. Grov & O. Tønder: Influence of IgG, $F(ab')_2$ and IgM on the phagocytic and bactericidal activities of human neutrophil granulocytes.

O. Tønder, E. C. Krishnan, W. R. Jewell, P. A. Morse & L. J. Humphrey: Tumor Fc receptors and tumor-associated immunoglobulins.

ADVICE TO AUTHORS

Authors must note and adopt the ACTA's customary arrangement and style; failure to do so may lead to delay in publication.

Instructions to authors are available on request to the Editorial Board. Manuscripts should be submitted to the national editor in their final form as top pages, not carbon copies, in double-spaced typescript in English, French, or German. All written matter, illustrations, and references should be submitted at the same time.

Ordinary articles should generally not exceed 5 printed pages and not more than 5 pages of illustrative material. They must contain a summary in English not exceeding 250 words. *Brief reports* for immediate publication must not exceed 1½–2 printed pages. Such reports will be published as soon as possible after receipt. Manuscripts will be reviewed by appropriate experts. Since manuscripts will not be insured against loss or damage, contributors are expected to retain duplicate copies of all material submitted for publication. Only illustrations of reasonable technical standards will be accepted. If the limit of 5 pages is exceeded and, if corrections in the proof are particularly numerous or the tabular and illustrative material unusually excessive and/or expensive, authors will be requested to contribute to the cost of publication. Reference to literature should conform to the standards of *World Medical Periodicals* or *World List of Scientific Periodicals*. In general, reviews of a topic unsupported by original observations will not be accepted.

The official abbreviation: Acta path. microbiol. scand. Sect. *A*, *B* or *C*.

Supplements: The publication of supplements will be governed by special rules which can be obtained from the Editor.

Articles accepted for publication in Section C

Jespersen: Potency of purified tuberculin determined by the shock method on hamsters.

Marker, G. Thörner Andersen & M. Volkert: The interplay between target organ concentrations of lymphocytic choriomeningitis virus and cell mediated immunity in baby mice.

-E. Svehag & D. Burger: Isolation of C1q-binding immune complexes by affinity chromatography and desorption with a diaminoalkyl compound.

Hanneberg & C. Endresen: Fragments of immunoglobulins in human feces.

Ødegaard & J. Lamvik: The effect of phenylbutazone and chloramphenicol on phagocytosis of radiolabelled *Candida albicans* by human monocytes cultured in vitro.

N. Wüg: Surface structures influencing the migration in vivo (ecotaxis) of murine thymocytes, T- and B-cells.

G Hansson: Evaluation of three reverse passive haemagglutination methods and two radioimmunoassay tests to be used for the detection of hepatitis B surface antigen.

Wanner, T. Midtvedt & A. Trippestad: Rat intestinal glycoprotein lowering bactericidal activity of serum on ³²P-labelled *E. coli*.

N Bruun, C. O. Solberg, E. Hanre, C. J. Jensen, S. Tvedt & J. Eide: Acute disseminated [illegible] in a patient with impaired neutrophil granulocyte function.

Midtvedt & A. Baardsen: Elimination of injected ³²P-labelled *E. coli* from rat polymorphonuclear neutrophils (PMN). Evaluation of a method.

O Solberg, K. E. Christie, B. Larsen & O. Tønder: Influence of antibodies and thermolabile serum factors on the bactericidal activity of human neutrophil granulocytes.

E Christie, C. O. Solberg, B. Larsen, A. Grov & O. Tønder: Influence of IgG, F(ab')₂ and IgM on the phagocytic and bactericidal activities of human neutrophil granulocytes.

Tønder, E. C. Krishnan, W. R. Jewell, P. A. Morse & L. J. Humphrey: Tumor Fc receptors and tumor-associated immunoglobulins.

ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA
Section C: Immunology

CONTENTS

Vol. 83 C. Fasc. 6. 1975